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INFLUENCE OF DIETARY FIBERS AND WHOLE GRAINS ON FECAL MICROBIOTA DURING IN VITRO FERMENTATION

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There has been a dramatic increase in obesity, which presents a risk of many chronic health problems. Recent studies have shown that obese individuals harbor an abnormal gut microbiota (dysbiosis), which has sparked interest in the gut microbiota as a target for weight management. Dietary fiber and whole grains that can be metabolized by gut microbiota have been shown to promote the growth of beneficial bacteria in the gut. Increased consumption of dietary fiber and whole grains may improve host / gut microbiota interactions in obesity and other metabolic diseases by normalizing gut dysbiosis. The present thesis describes two research projects to assess the impact of dietary fibers and whole grain on gut microbiota. In the first study, six dietary fibers [pectin, guar gum, inulin, arabinoxylan, β-glucan, and resistant starch type 2 (RS-2)] were subjected to *in vitro* digestion and fermentation using fecal samples from obese and normal weight individuals. Pyrosequencing was used to assess the impact of each dietary fiber on the gut microbiota community. Short/branched chain fatty acids (SCFA/BCFA) and carbohydrate utilization were correlated with proportions of bacterial taxa. The data showed that RS-2 caused the most dynamic change of the whole microbiota community and *Bifidobacterium* increased almost 10-fold on pectin substrate compared with the control. Certain taxa may be targeted to increase SCFA production or increase dietary fiber utilization. For instance, *Ruminococcaceae* and *Faecalibacterium* displayed positive correlations with butyrate production and while a strong positive relationship was shown between β-glucan utilization and *Firmicutes.* In the second study, since SCFA can influence hormones involved in energy absorption, utilization, and storage, the SCFA profile from five whole grains (wheat, rye, corn, rice, and oats) using an *in vitro* method

was evaluated. There were large differences in fermentation profiles among individuals, even when supplied with the same dietary fiber. The obese type microbiota was less efficient at butyrate production and less metabolically active than the normal weight type microbiota initially, but given sufficient whole grain substrates the bacteria quickly became metabolically active.

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INTRODUCTION

About one-third of U.S. adults (33.8%) are obese. Obesity is the key contributor to many metabolic syndromes worldwide such as hypertension, dyslipidemia, type 2 diabetes, colon cancer, cardiovascular disease and stroke [1].

Human intestines harbor an immensely complex and diverse microbiota that can be viewed as a super metabolic organ (15-fold more genes than our host genome) with about 10^{14} bacteria and Achaea, composed of approximately 1,100 prevalent species [2]. The gut microbota has been found to affect energy harvest from the diet and fat storage [3]. Germ-free mice are protected from diet induced obesity, and upon gut microbiota colonization a significant increase (42%) of body fat content occurs [4].

Gut microbiota composition has been associated with obesity and many metabolic diseases. Studies [5-7] show that there is a shift between two bacterial phyla (*Bacteroidetes* and *Firmicutes* in obese state): a reduction of *Bacteroidetes* and a proportional increase of *Firmicutes* in the process of weight gain. Also compared with lean people, there are relatively less *Bacteroidetes* in obese people, which are increased with weight loss [8, 9].

The gut microbiota can be influenced by diet, particularly by dietary fibers and whole grain in the diet. Indeed, certain dietary fibers can favor the growth of one or a group of bacteria at the expense of others [10]. Moreover, obese people consistently consume less dietary fiber than normal-weight individuals [11].

Objectives and Hypotheses

We hypothesized that whole grain and dietary fibers may be an excellent way to treat the obesity by altering and modulating of the gut microbiota. Since whole grains and dietary fibers are actually composed of many chemical compounds with different physiological effects, we further hypothesized that different whole grains and dietary fibers would exhibit varying efficacy with respect to correcting the abnormal gut microbiota in obesity. Thus, the overall objective of this research was to discover the specific types of dietary fiber and whole grain that are most

efficient at altering the fecal microbiota composition or metabolism from obese individuals towards a composition and function more indicative of normal-weight individuals in an effort to identify candidate dietary fibers that will help prevent or treat obesity.

Organization

This thesis is organized as follows: a literature review (Chapter 1) followed by manuscripts describing two research projects (Chapters 2 and 3). Chapter 1 has been formatted using the guidelines for *Critical Reviews in Food Science and Nutrition*; Chapter 2 for *Applied and Environmental Microbiology*; and Chapter 3 for *Anaerobe*. Reference can be found at the end of each chapter.

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CHAPTER 1. INFLUENCE OF WHOLE GRAINS AND DIETARY FIBER ON THE GUT MICROBIOTA AND OBESITY

1.1. Abstract

There has been a dramatic increase in obesity, which presents a risk of many chronic health problems. Recent evidence suggests that obese people have a different gut microbiota composition compared to normal weight individuals and the gut microbiota is involved in nutrient absorption and energy balance. The proposed mechanisms that describe the influence of the gut microbiota on obesity include: 1) modulating energy harvest from the diet, 2) influencing energy and fat utilization, and 3) modulating low grade inflammation. A number of epidemiological studies have established that high dietary fiber / whole grain diets are associated with weight loss, suggesting that whole grains and dietary fibers may be important candidates to modulate the gut ecosystem to a healthier state and treat obesity. There are diverse types of dietary fiber and whole grain with varying structures that may have differing physiological impacts on the gut microbiota and obesity prevention.

1.2. Introduction

Globally, 300 million people are obese and more than 1 billion are overweight (Stephenson et al., 2010). In the US, one third of adults (33.8%) are obese (Flegal et al., 2010). Obesity has been viewed as one of the major public health problems in both adults and children, leading to conditions such as heart disease, stroke, type 2 diabetes, and certain types of cancer (Musso et al., 2010).

The underlying reasons contributing to obesity are complex. Although it essentially results from energy imbalance involving consuming more calories than expended, genetic background and environmental factors also play a role. Notably, epidemiological studies suggest that the recent increased prevalence in obesity cannot solely be explained by genetics, food availability, and behavioral changes (Raoult, 2008). The human microbiota has been recently

identified as a contributing factor to obesity, with different compositions observed between lean and obese individuals (Ley et al., 2005; Ley et al., 2006a; Ley et al., 2006b). The altered gut bacteria in obese individuals, or “dysbiosis”, may lead to increased energy absorption and storage (Mandard, 2005).

Dietary fiber and whole grains that can be metabolized by the gut microbiota have been shown to promote the growth of beneficial bacteria in the gut (Liu, 2007; Quigley, 2011). Increased consumption of dietary fiber and whole grains may improve host / gut microbiota interactions in obesity and other metabolic diseases by normalizing the dysbiosis of the gut microbiota. Because the diverse compositions and structures of dietary fibers and whole grains lead to differing impacts on the gut microbiota (Snart et al., 2006; Abell et al., 2008; Mitsou et al., 2010), some dietary fibers or whole grains may be more beneficial against obesity than others (Andersson et al., 2010).

In this article, physiological interactions between the gut microbiota and the host will be reviewed, followed by a discussion on the role that dietary fiber and whole grains may play in improving the composition and function of the gut microbiota in obesity.

1.3. Gut microbiota composition and contribution to obesity

There are at least 10^{14} microorganisms in the human colon belonging to over 1000 species (Egert et al., 2006). Although the gut is essentially sterile at birth, it is rapidly populated with bacteria through environmental exposure (Palmer et al., 2007). Facultative and aerobic bacteria establish first, but are later overcome by more strict anaerobes as the environment in the colon becomes more reduced (Adlerberth and Wold, 2009). Ecological and evolutionary forces shape the microbial diversity in the human gut and lead to a stable microbiota in adults that is dominated by members of two bacterial phyla: *Firmicutes* and *Bacteroidetes* (Sekirov et al., 2010; Ley et al., 2006a; Neish, 2009).

Bacteriodetes and *Firmicutes* account for 90% of the total bacteria that are amplified from 16S RNA sequencing (Ley et al., 2006b). *Bacteriodetes* is a diverse gram negative bacterial phylum including four classes: *Bacteroidia, Flavobacteria*, *Sphingobacteria*, and *Cytophagia*, representing around 7000 different species. Most of them evolve with the host into a symbiotic relationship, functioning to degrade indigestible dietary carbohydrates (Michel et al., 2011) and host-derived carbohydrates such as mucins (Salyers et al., 1977). Short chain fatty acids (SCFAs), especially butyrate and succinate, are produced as the main fermentation metabolites, which play important roles in gut health (Kim and Milner, 2007). Some species in this phylum, such as *Bacteroides fragilis*, also secrete various proteases (Gibson and Macfarlane, 1988), which result in undesirable fermentation products such as ammonia, hydrogen sulfide, and phenol (Macfarlane and Macfarlane, 2012). *Firmicutes* is a gram positive phyla containing 3 classes: *Bacilli, Clostridia*, and *Mollicutes*, and 164 genera. Clostridial cluster IV and XIVa, which constitute more than 80% of total bacteria in this phyla (Duncan et al., 2007), are abundant fermenters of starch, fructans, and cellulose, with production of acetate, butyrate, and lactate. *Peptococcus, Peptostreptococcus* and *Clostridum* are the predominant proteolytic genera in this phylum (Dimitris Charalampopoulos and Rastall, 2009).

The association between obesity and the gut microbiota composition was initially established with mouse models. Genetically obese, leptin-deficient mice showed 50% fewer *Bacteroidetes* accompanied by a proportional increase in *Firmicutes* compared with wild type counterparts (Ley et al., 2005). By exploring the effects of high-fat diets and genetic obesity on the gut microbiota over time, Murphy et al. (2010) found an increase in *Firmicutes* and decreases in *Proteobacteria* and *Bifidobacterium* spp. in both high-fat-fed wide-type and genetic ob/ob mice. Hildebrandt et al. (2009) reported that mice fed a high-fat diet for three months have higher *Clostridiales* and fewer *Bacteroidetes*.

Since these discoveries, a number of studies have reported the differences in gut microbiota composition in obese states compared with normal weight states in humans (Table

1.1). Consistent with the mouse model data, the first human studies showed a higher proportion of *Bacteroidetes* in normal weight individuals compared with obese (Ley et al., 2006b; Schwiertz et al., 2009); however, others have reported more *Bacteriodetes* and an enrichment of hydrogen-producing *Prevotellaceae* in obese individuals compared with normal weight (Zhang et al., 2009). Furet et al. (2010) showed lower *Bacteroides-Prevotella* and *Faecalibacterium prausnitzii* in obese subjects, while another study showed significantly higher *Bacteroides* in overweight women compared with normal-weight women (Collado et al., 2008).

Upon weight loss in obese individuals, changes in the gut microbiota have been reported but are also not consistent (Table 1.1). For instance, Schwiertz et al. (2009) showed that the proportion of *Bacteroidetes* is reduced by weight loss. In contrast, Duncan et al. (2008) reported no change in *Bacteroidetes* after 4 weeks on a reduced carbohydrate diet, but did report a significant reduction in a group of butyrate producing *Firmicutes* in the obese group after diet intervention. In other studies, higher *Bacteroides* was associated with excessive weight gain (Collado et al., 2008), and weight loss was accompanied by an increase in *Bifidobacteria* (Santacruz et al., 2010).

Many of these findings above were reported using methods that only amplify specific bacteria in the community. Thus, changes or shifts may have been present but not discovered or reported. Additionally, these methods have some inherent drawbacks that render the results only semi-quantitative. Accompanied by the complexity of the gut microbiota and the large differences among individuals, this may explain some of the inconsistencies in the gut microbiota composition in obesity and changes upon weight loss.

1.4. Proposed mechanisms for the role of gut microbiota in obesity

1.4.1. Modulating energy harvest from diet

Compared with germ-free mice, conventional mice exhibit a greater ability to harvest energy from food, contributing to body weight gain (Backhed, 2004). Transferring the fecal

microbiota from obese mice into wide type, germ-free mice results in increased fat gain and dietary calorie extraction in the recipient compared to when fecal microbiota from normal weight mice are used, indicating more efficient energy extraction by the obese microbiota (Turnbaugh et al., 2006).

A meta-genomic study showed that the gut microbiota are enriched in the genes involved in energy harvest in obese mice compared with lean mice (Lakhan and Kirchgessner, 2011). An enhanced expression hydrolases and lysases would lead to metabolism of undigested material that would, under normal circumstances, be inaccessible (Musso et al., 2010). This would result in higher SCFA production in obese states compared with normal weight, which can contribute to extra energy recovery for the host (Turnbaugh et al., 2006; Schwiertz et al., 2008), as well as reduced fecal energy content (Webb and Annis, 1983; Turnbaugh et al., 2006).

This finding is peculiar considering that colonic fermentation of indigestible carbohydrates with accompanying SCFA production is generally recognized as beneficial to gut health (Campbell et al., 1997; Tedelind et al., 2007). Of note is that epidemiologic studies have consistently reported less dietary fiber intake in obese individuals compared with normal weight (Miller et al., 1994; Alfieri et al., 1995; Koh-Banerjee et al., 2004; Davis et al., 2006; Ben Slama et al., 2011). Although speculative, it may be that in obese states the gut microbiota are constantly starved for substrate and express more diverse enzymes so as to utilize every last bit of substrate available, even those that may result in undesirable fermentation products (Le Leu et al., 2006; Macfarlane and Macfarlane, 2012), leading to dysbiosis. Conversely, the relative abundance of dietary fiber substrate for normal weight individuals consuming adequate dietary fiber render the microbiota more selective in what they metabolize, leading to more beneficial fermentation products and higher fecal energy.

Furthermore, although SCFAs contribute about 10% of total energy to the diet (McNeil, 1984), they also influence hormones, which, in many cases, increase the feeling of satiety (McBurney et al., 1990; Tucker and Thomas, 2009), reduce absorption of energy, or reduce

energy storage (Table 1.2). For instance, SCFA (especially propionate and acetate) are ligands for GPR41, identified as a G protein-coupled receptor and expressed by a subset of enteroendocrine cells in the gut epithelium (Samuel et al., 2008). GPR41 expressed in human colonic mucosa may function as a sensor for luminal SCFAs (Tazoe et al., 2009). Upon ligand binding, these receptors stimulate secretion of the peptide hormones PYY and leptin that modulate gut motility and reduce energy harvest from diet. GPR41-deficient mice show a reduced expression of PYY (Samuel et al., 2008). Obese individuals have decreased serum leptin levels associated with increased hunger and reduced energy expenditure. Modulation of GPR41 is a potential therapeutic target to control energy extraction.

Glucagon-like peptide-1 (GLP-1) is a key hormone released from enteroendocrine L-cells in the ileum and proximal colon (Drucker, 2006). Administration of GLP-1 can increase secretion of insulin from pancreatic b-cells (Baggio and Drucker, 2007) and induce satiety and weight loss in animals and humans. Increased fermentation of indigestible carbohydrate in the colon is associated with higher plasma GLP-1 by promoting L-cell differentiation in proximal colon (Turton et al., 1996; Piche et al., 2003; Cani et al., 2004; Cani et al., 2005). Some have postulated that this may be due to SCFA production from indigestible carbohydrate fermentation (Cani et al., 2007b). It has been shown that butyrate enhances proglucagon gene expression and GLP-1 content in immortalized colonic L-cells *in vitro* (Dumoulin et al., 1995; Pratt et al., 1996), and the SCFA receptor GPR43 is expressed on L-cells (Karaki et al., 2006). Therefore, sufficient production of butyrate might help increase GLP-1 production and be conductive to controlling body weight.

The gut microbiota can modulate fat emulsification and absorption as well as lipid metabolism by influencing bile acid metabolism. In the small intestine, bile acids promote emulsification and micelle formation of dietary lipids and lipid-soluble vitamins, which is required for absorption (Ridlon, 2005). Approximately 400–800 mg of bile salts per day make their way to the colon and can be biotransformed by microbial enzymes and then delivered to the

liver where they become part of the bile acid pool (Matern and Gerok, 1979). Martin et al. (2007) found that, compared with conventional gut microbiota, colonization with human intestinal microbiota strains (high in *Bacteroides* and *Enterobacteria)* was correlated with changes in the bile acid pattern toward tauro-conjugated bile acids and higher absorption of dietary lipids. Tauro-conjugated bile acids are more efficient at lipid emulsification and micelle formation compared with deconjugated bile acids (Armstrong and Carey, 1982; Heuman, 1989). They also found that *Lactobacillus*, *Bifidobacteria* and *Enterobacteria*, and *Bacteroides* might play an important role in the deconjugation process, thus resulting in less efficient dietary fat emulsification and absorption (Grill et al., 1995). Moreover, normal bile acids (i.e., unconjugated) have been associated with increased energy expenditure by promoting intracellular thyroid hormone activation (Watanabe et al., 2006).

1.4.2. Influencing energy and fat utilization

Gut microbiota also modulate energy and fat deposition by several pathways. Fast induced adipose factor (Fiaf), or angiopoietin-like protein 4, was first identified as a target gene of the nuclear receptor peroxisome proliferator-activated receptors (PPAR), which can stimulate fatty acid oxidation and prevent fat storage in both muscle and adipose tissue (Mandard, 2005). Backhed et al. (2007) demonstrated that germ free knockout mice lacking Fiaf are not protected from diet-induced obesity and colonizing germ-free mice with gut microbiota leads to decrease the expression of Fiaf.

Another enzyme related to fat oxidation and storage is AMP-activated protein kinase (AMPK), which may initiate fatty acid oxidation in peripheral tissues of muscle and liver by phosphorylating acetyl CoA carboxylase and decreasing glycogen storage (Mandard, 2005). Active-AMPK is 40% higher in germ free animals compared with conventional animals. The gut microbiota can suppress skeletal muscle fatty acid oxidation in an AMPK-related metabolic pathway, although the exact pathway whereby the microbiota signals AMPK remains unclear

(Tilg and Kaser, 2011). Thus, the gut microbiota may play a role in controlling energy homeostasis and nutrient availability.

1.4.3. Modulation of low grade inflammation

Obesity has been linked to low grade chronic inflammation (Wellen, 2005). Bacterial lipopolysaccharide (LPS), which is produced upon the death and translocation of gram-negative bacteria in the gut, has been identified as a triggering factor in the onset of systemic inflammation (Cani et al., 2007a). An increase in plasma LPS concentration could be induced by high-fat feeding. Mice fed for 4-weeks with a high-fat diet had plasma LPS levels two to three times higher than normal, and exhibited obesity and insulin resistance accompany by metabolic endotoxemia (Cani et al., 2007a). *Bifidobacteria* have been shown to reduce intestinal LPS and fortify intestinal barrier function, indicating that composition of gut microbiota is related to inflammation and obesity (Griffiths et al., 2004).

Besides the mechanisms discussed above, it has also been proposed that the gut microbiota could decrease body weight by placing an additional energetic burden on the host. The underlying mechanism may involve stimulating synthesis of new gut tissue, adjusting inflammation or activating host defense system (Flint, 2011). Nevertheless, it is increasingly clear that the gut microbiota play roles in energy hemostasis and weight loss, although the exact roles and the specific microbial taxa involved remain elusive.

1.5. Dietary intervention as a treatment to modify the gut microbiota in obesity

A number of studies (Duncan et al., 2008; Santacruz et al., 2009) have shown that the diet is an important and fundamental promoter of differences in gut bacterial composition and diversity. Dietary fiber serves as the main source of energy for gut bacteria, and dietary intervention can change the microbiota composition to a healthier state (Jenkins et al., 1987). Thus, dietary fiber and foods high in dietary fibers, such as whole grains, fruits, and vegetables, are intriguing candidates for correcting the dysbiosis in obese states and promoting weight loss.

1.5.1. Dietary fiber

In 2008, the Codex Commission on Nutrition and Foods for Special Dietary Uses (CCNFSDU) gave the definition of dietary fiber as carbohydrate polymers with 3-9 monomeric units, which are not hydrolyzed by endogenous enzymes in the small intestine of human beings and belong to the following three categories: 1) edible carbohydrate polymers naturally occurring in food, 2) carbohydrate polymers that have been obtained from raw material in food by physical, enzymatic, or chemical means and which have been shown to have physiological of benefit to health, and 3) synthetic carbohydrate polymers that have been shown to have physiological effect of benefit to health (Raninen et al., 2011).

Dietary fiber can be divided into two classes based on solubility in water: soluble dietary fiber and insoluble dietary fiber. Pectin, guar gum, soluble β-glucan, and polysaccharide gums are examples of soluble dietary fiber. Soluble dietary fiber shows favorable effects on controlling glucose level and lipid metabolism homeostasis, mainly due to an increased viscosity in luminal contents (Galisteo et al., 2008). Hemicellulose, cellulose and lignin are among the common insoluble dietary fibers, which promote fecal bulking, softening and laxation. Both soluble and insoluble dietary fibers can be fermented by gut microbiota in colon and yield SCFAs, although soluble generally results in comparatively more rapid and higher concentrations of SCFAs.

According to the International Scientific Association of Probiotics and Prebiotics, “a dietary prebiotic is a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota; thus conferring benefit(s) upon host health” (Ley, 2005). Most research has been focused on selective stimulation of *Bifidobacteria* and *Lactobacilli* by oligosaccharides (Depeint et al., 2008; Holma et al., 2002; Kolida et al., 2002). These products may also result in beneficial physiological effects not only in colon, but systemically through prevention of inflammation (Ewaschuk and Dieleman, 2006), immune modulation (Bodera, 2008), and energy metabolism (Woods, 2005).

Epidemiological studies show a significant inverse association between dietary fiber consumption and body mass index (BMI; McBurney et al., 1990; Tucker and Thomas, 2009). In 203 healthy men, intakes of complex carbohydrate and dietary fiber were associated with lower body fat, especially in the highest body fat groups (Nelson and Tucker, 1996). In another study, higher dietary fiber consumption was correlated with reduced body weight and fat in women (Tucker and Thomas, 2009). Combined with other studies, epidemiological studies convincingly indicate an inverse association between dietary fiber intake and body weight (Howarth et al., 2001).

A number of mechanisms describing how dietary fiber might reduce obesity have been proposed. Dietary fiber can promote the feeling of satiety compared with starch and simple sugars (Connolly et al., 2010; Dunne et al., 2001; Pereira and Ludwig, 2001). Increasing satiety may result from several factors including the intrinsic properties of dietary fiber (high viscosity, bulking and gel formation), which can reduce the rate of gastric emptying and macronutrient absorption, and effects on certain gut hormones [PYY, ghrelin and cholecystokinin (CCK)] that can signal satiation (Woods, 2005). Additionally, high fiber foods are less energy dense compared with high fat and high protein food. Thus, high fiber foods can displace energy so as to reduce body weight. High fiber foods in general also do not promote rapid fluctuations in blood glucose levels (Giacco et al., 2000).

In spite of the convincing role of dietary fiber in preventing obesity suggested by epidemiological studies and proposed mechanisms, intervention studies on weight loss and dietary fibers intake are inconsistent. Birketvedt et al. (2000) found that increased dietary fiber intakes were associated with body weight loss, while, in a pilot study (Howarth et al., 2003), dietary administration of fermentable and non-fermentable fiber did not change body weight. Moreover, other intervention studies have failed to demonstrate a clear change in satiety upon dietary fiber supplementations (Smith and Tucker, 2011). One mechanism that has not been

thoroughly explored is the influence of dietary fibers on the gut microbiota in obesity and how they may modulate the microbiota to a composition that is more conducive to weight loss.

Different dietary fibers show quite different fermentation impacts on gut bacteria. For instance, *Bifidobacteria* and *Lactobacilli* cannot ferment cereal β-glucan alone *in vitro* but can utilize oligosaccharides resulting from its partial hydrolysis (Crittenden et al., 2002). In contrast, *Bifidobacterium longum* and *Bifidobacterium adolescentis* can ferment long chain arabinoxylan (AX) *in vitro* from a variety of cereals without pre-hydrolysis. It is likely that different dietary fibers are more effective than others at modulating obesity since dietary fiber is a complex class of compounds (Table 1.3).

Fructans

Fructan is a general term for a wide variety of carbohydrate such as fructooligosacchrides (FOS), oligofructose, and inulin, depending on degree of polymerization (DP). Fructans reach the large colon and undergo fermentation by gut microbiota. FOS with low DP are mostly fermented in the proximal colon, while some inulin and fructans with longer DP may reach more distal regions of the colon (Alles et al., 1996). The primary effect of inulin type prebiotics on gut ecology is the stimulation of bifidobacterial growth (Roberfroid, 2007). By adding 15 g inulin/d to the diet for 15 days, *Bifidobacteria* increase by 1 log compared to the baseline (Liu, 2007). Inulin-type fructans may have an influence on weight loss, although research in this area is very limited. One dietary intervention study with 97 adolescents demonstrated a significant reduction in weight gain for the people receiving 8g/d oligofructose-enriched inulin compared with maltodextrin as a control for a year; the difference disappeared one year after the supplementation was stopped (Abrams et al., 2007).

Resistant starch

Some starch may escape digestion in the human small intestine and reach the colon to be fermented by gut bacteria. Resistant starch (RS) can be classified into 4 main types: physically inaccessible starch (RS-1), resistant granules and high amylose starches (RS-2), retrograded

starches (RS-3) and chemically modified starches (RS-4; Bird et al., 2000). Fermentation of RS, especially by RS-2, has been shown to increase the production of butyrate. However, Ferguson et al. (2000) showed that fermentation of resistant starch from potato yields more butyrate than high amylose corn starch. Another study showed that RS-4 but not RS-2 leads to profound phylum-level changes, significantly increasing *Actinobacteria* and *Bacteroidetes* while decreasing *Firmicutes* (Martínez et al., 2010). These studies indicate that differences exist in the fermentation properties of different type of RS, even though they are the same chemical structure. With regard to obesity, some studies show that inclusion of RS in the diet can reduce body fat by energy dilution and changing expression of PYY and GLP-1 (Keenan et al., 2006; Shen et al., 2008). Another study found that, compared with the control (0% resistant starch), administration of 5.4% resistant starch significantly increased lipid oxidation (Higgins et al., 2004), thereby reducing the fat accumulation.

Arabinoxylan

AXs are the most abundant non-digestible carbohydrates present in most cereals. AX can be divided into water-extractable (WE-AX) and water-unextractable (WU-AX) according to extractability in water, as well as arabinoxylan oligosaccharides (AXOS), which are hydrolysis products of AX. Branching, molecular weight, and cross-linking with other components contribute to their solubility (Neukom, 1973). AXs are broken down in the colon by intestinal bacteria that express AX-degrading enzymes such as xylanases and arabinofuranosidases (Hughes et al., 2007). Fermentation of AX by human intestinal bacteria *in vitro* is associated with a higher proportion of propionate production compared with other dietary fibers (Amrein et al., 2003; Van Laar et al., 2002). High fat mice supplemented with WE-AX, have shown increases in clostridial cluster XIV, *Bacteroides-Prevotella* spp., and *Bifidobacteria* accompanied by reduced circulating inflammatory markers, body weight, and hepatic cholesterol-lowering effects (Brennan et al., 2011). WE-AX of different molecular weights and cross-linking can affect selectivity toward certain bacterial groups. Fermentation of a 66 KDa WE-AX from wheat particularly aided in the

growth of *Lactobacilli* and *Eubacteria,* while other WE-AX fractions (278, and 354 KDa) only increased *Eubacteria* (Hughes et al., 2007). WE-AX cross-linked with ferulic acid resulted in a slower fermentation rate than non-cross-linked WE-AX, the latter of which has also been correlated to increases in *Bacteroides fragilis in vitro* (Hopkins et al., 2003). WU-AX are only partially fermented in the colon, but stimulate butyrate and butyrate-producing bacteria such as *Roseburia/E. rectale* spp. (Damen et al., 2011). Fermentation of AXOS can reduce the pH and significantly increase the *Bifidobacteria* more so than WE-AX and WU-AX. Some research suggests that fermentation of AXOS has stronger ability to stimulate *Bifidobacteria* than fructans (Hsu et al., 2004).

β-Glucan

Mixed linkage (1, 3 and 1, 4) β-glucans are a ubiquitous group of nonstarch polysaccharides in cereal grains, but are found in the highest concentrations in oats and barley (El Khoury et al., 2011). Other sources of β-glucan are the cell walls of yeast, fungi, and some bacteria (Volman et al., 2008). β-Glucan has been demonstrated in clinical trials to significantly impact weight loss by increasing satiety, influencing absorption efficiency in small intestine and lowering cholesterol (Huang et al., 2011; Kim et al., 2006; Rondanelli et al., 2011). In addition, β-glucan has been documented to increase insulin sensitivity (Ximenes et al., 2007). The health benefits of β-glucan in the gastrointestinal tract, such as increasing satiety and reducing blood serum cholesterol and blood glucose, are correlated with molecular weight distribution and viscosity. The few studies evaluating the influence of β-glucan on the gut microbiota have suggested that it exhibits only moderate influences on the gut microbiota compared with prebiotics such as inulin (Hughes et al., 2008; Jha et al., 2011; Lin et al., 2011; Mitsou et al., 2010; Pieper et al., 2008). However, an increase in *Bifidobacteria* compared with none detected at baseline and significant increase of *Bacteroides* were observed by dietary intervention with β-glucan (Mitsou et al., 2010).

1.5.2. Whole grain

Evidence continues to mount that whole grains can protect people from many chronic diseases including obesity, cardiovascular disease, and type-2 diabetes (Quigley, 2011). Whole grains are defined as “intact ground cracked or flacked caryopsis, whose principal anatomical components—the starchy endosperm, germ, and bran—are present in the same relative proportions as they exist in the intact caryopsis” (Force, 1999). The Whole Grains Council notes that, “if the grain has been processed (e.g. cracked, crushed, rolled, extruded, and/or cooked), the food product should deliver approximately the same rich balance of nutrients that are found in the original grain seed” (WGC, 2004).

Epidemiological studies have found there is an inverse relationship between consumption of whole grain and obesity. For instance, in three studies based on food frequency questionnaires encompassing 3559 participants, whole grain intake was inversely related to body weight gain and waist-to-hip ratio (McKeown et al., 2002; Newby et al., 2003; Rose et al., 2007b). Increased consumption of whole grain was also correlated with lower BMI and waist circumference in the analysis of the data from 1999-2004 NHANES containing 13,000 adults from 19-50 years old (O'Neil et al., 2010). In a study using nationally representative samples of adults living in Great Britain from 1986–1987 and 2000–2001, an inverse association between whole grain intake and obese population was observed in 1986-1987 (Thane et al., 2007). Another study that compared whole and refined grain consumption showed that both subcutaneous and visceral adipose tissue were inversely associated with whole grain intake, while positively associated with refined grain intake (McKeown et al., 2010).

Data from intervention studies have not totally supported results from epidemiological studies. Katcher et al. (2008) examined 50 obese men and women with metabolic syndrome who received whole grain cereal for 12 weeks and showed significant decreases in both body weight and percent body fat. However, in an intervention study on 64 healthy men and women for 4 weeks, no relationship between whole grain cereal consumption and body weight loss was

observed (Waller et al., 2004). Furthermore, Brownlee (2009) did not find a significant association between whole grain intake and body weight when 361 overweight participants were supplemented with whole grains in the diet (60 -180 g/day).

Mechanisms have been proposed relating to the protection of whole grains against obesity. These include the influence of the dietary fiber component discussed above, but also include the influence of antioxidants and other phytochemicals present in whole grain that have been shown to be involved signaling for many metabolism pathways (Fardet, 2010).

Influence of whole grain on the gut microbiota in obesity is an unexplored area of research. Whole grain intake has been associated with reduced distal colonic pH (Sengupta et al., 2001). This may be due to the prevalence of insoluble dietary fiber in whole grains. Insoluble dietary fiber is largely responsible for supporting bacterial metabolism in the distal colon due to its slower fermentation rate (Leach, 2006). This may be important for preventing low-grade inflammation in this region, which is a factor in obesity.

Dietary fiber from whole grain can impact gut microbiota. For instance, *in vitro*, *Bifidobacterium longum* and *B. adolescentis* can ferment AX (Crittenden et al., 2002), while these substrates cannot be fermented by potentially harmful bacteria such as *Escherichia coli*, *Clostridium perfringens*, or *C. diffcile* (Oikarinen et al., 2007). Cellulolytic bacteria such as those belonging to clostridial cluster IV have been shown to possess the enzymes and ability to degrade cellulose (a major component in whole grains). Recently discovered species, *Bacteroides cellulosilyticus*, has the strong ability to break down insoluble cellulose (Robert and Bernalier-Donadille, 2003; Robert et al., 2007; Wedekind et al., 1988), producing SCFA and lowering the pH in the colon. Therefore, whole grains may have their unique health benefits including prolonging / sustaining the fermentation profile and selectively stimulating certain bacteria by providing fermentable substrates to the gut microbiota.

Notably, all whole grains are not identical (Table 1.4) and thus may result in different physiological effects. If one whole grain is more effective at preventing obesity than others,

recommendations for food intake could be tailored to include foods high in this grain for prevention or treatment of obesity. For instance, Andersson et al. (2010) fed mice whole grain wheat or whole grain rye for 22 weeks and found that rye reduced body weight, adiposity, and total plasma cholesterol and improved insulin sensitivity compared with wheat. This may be because rye is the highest in water extractable AX among other cereal grain, since AX supplements have been shown to decreased adipocyte size and inflammation (Neyrinck et al., 2011).

Grains also contain unique bioactive phytochemicals including derivatives of benzoic and cinnamic acids, anthocyanidins, quinones, flavonols, chalcones, flavones, flavanones, and phenolic compounds (Adom and Liu, 2002). It has been shown that these antioxidants might protect the gut epithelial cell, prevent inflammation, and reduce the intensity of diabetes (Balasubashini et al., 2004; Slavin, 2007). Most of these components are present in the germ/bran fraction. For instance, 83% of total phenolic content, 79% of total flavonoid content are concentrated in the bran/germ fraction of wheat (Liu, 2007). Most of these compounds are bound to cell wall material and can resist digestion in the small intestine and reach colon, where gut microbiota can convert these compounds to their metabolites such as phenolic acids or lactone structures (Aura, 2008). Andreasen et al. (2001) demonstrated that human and rat colonic microbiota have the ability to release diferulic acids from dietary cereal bran. Upon releasing, ferulic acids rapidly become bioavailable to the host, resulting in alleviating oxidative stress during inflammation such as in obesity (Huang F et al., 2011).

Whole grains can contain other bioactive components that may affect physiological properties. For instance, wheat bran contains at least twice as much betain than oats, while corn contains no betain. Betain has been linked to metabolic syndrome, lipid disorder and diabetes (Lever and Slow, 2010; MacKay et al., 2010). Oats contain unique, anti-inflammatory compounds called avenanthramides have been shown to reduce proliferation of colonic cancer cell (Guo et al., 2010). Although the protective effect of avenanthramides on obesity remains

unknown, avenanthramide extracts from oats have been shown to inhibit LDL (low density lipoprotein) oxidation, which protects against atherosclerosis (Dykes and Rooney, 2007).

1.6. Conclusion

Recent evidence suggests that both compositional and functional differences in gut microbiota exist in lean and obese individuals. The human gut microbiota can influence energy harvest and energy expenditure of the host. Recent mechanistic studies in animal and human models have provided insight into the proposed contributory role of the gut microbiota in energy metabolism and weight control. Modulation of the gut microbiota composition or its biochemical capacity may be facilitated by dietary intervention. Considering the unique chemical and physiological properties of whole grains and dietary fiber, these compounds may serve as important candidates for dietary intervention to elicit beneficial effects especially by altering obese type gut microbiota to a healthier state. Research initiatives to establish the interrelationship among whole grains and dietary fibers, the gut microbiota, and the metabolism of the host are needed.

1.7. References

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Table 1.1. Differences in fecal gut microbiota composition in obese and normal weight individuals.[a]

Subjects	Dietary intervention	Method	Differences between normal weight and obese	Changes upon weight loss in obese subjects	Reference
12 obese participants for one year (2 lean controls)	On a fat restricted diet (30% calories from fat) or carbohydrate restrict diet (25% calories from carbohydrate) with total calories 1200-1800 kcal/d.	16S rRNA sequencing	Higher *Firmicutes: Bacteroidetes* ratio in obese compared with lean individuals at baseline	Increase in proportion of *Bacteroidetes* with weight loss	Ley et al., 2006
15 obese and 14 normal weight for 8 weeks	Reduced carbohydrate weight-loss diet	FISH	No difference between BMI and relative population of *Bacteroidetes*	Significant reductions in a group of butyrate-producing *Firmicutes* in obese subjects on weight lose diet; no association between weight loss and population of *Bacteroidetes*	Duncan et al., 2007
18 overweight and 36 normal weight women	No intervention	FISH and RT-PCR	*Bacteroides* and *Staphylococcus* were significantly higher in the overweight state than in normal-weight women		Collodo et a al ., 2008
25 obese; 25 overweight; 24 normal weight children at 7 years of age	No intervention	RT-PCR	More *Bifidobacteria* in infancy contribute to normal weight; overweight was related to more *Staphylococcus aureus* in the fecal sample during infancy		Kalliomaki et al., 2008

Table.1.1 (continued). Differences in fecal gut microbiota composition in obese and normal weight individuals.[a]

Subjects	Dietary intervention	Method	Difference between normal weight and obese	Changes upon weight loss in obese subjects	Author
39 obese and normal weight individuals for 10 weeks	Energy restrict diet (calorie reduction =10–40%) and a physical activity program	FISH		Reduction of *E. rectale-C. coccoides* and *C. histolyticum* proportions with weight loss	Nadal et al ., 2009
23 subjects (BMI=29.4-35.2)	Calorie-restricted diet (calorie reduction =10-40%) and increased physical activity	RT-PCR		Increased *Bacteroides fragilis* and *Lactobacillus* groups, decreased *Clostridium coccoides* and *B. longum* in high weight loss compared with moderate weight loss group	Santacruz et al ., 2009
33 obese; 35 overweight; 30 lean individuals	No intervention	RT-PCR	*Firmicutes:Bacteroidetes* ratio was lower in overweight and obese compared with normal weight		Schwiertz et al ., 2009
3 morbidly obese(BMI>35); 3 normal weight; 3 post gastric bypass surgery	No intervention	454 pyrosequencing /RT-PCR	More *Bacteriodetes* in obese than normal individuals; *Prevotellaceae* enriched in obese subjects compared with normal weight		Zhang et al ., 2009

Table.1.1 (continued). Differences in fecal gut microbiota composition in obese and normal weight individuals.[a]

Subjects	Dietary intervention	Method	Difference between normal weight and obese	Changes upon weight loss in obese subjects	Author
30 obese individuals with type-2 diabetes and 13 lean control	No intervention	RT-PCR	*Bacteroides-Prevotella* and *Faecalibacterium prausnitzii* were lower in obese subjects		Furet et al ., 2010
16 overweight and 34 normal weight	No intervention	RT-PCR	*Bifidobacterium* and *Bacteroides* were inversely associated with body weight, whereas an opposite trend was found for *Staphylococcus*, *Escherichia coli*, and *Enterobacteriaceae*	More *Bifidobacterium* in women with normal weight gain than excessive weight gain during pregnancy	Santacruz et al ., 2010

[a]Normal weight [body mass index (BMI) <25]; overweight (25<BMI<30); obese (BMI>30); FISH: fluorescence *in situ* hybridization.

Table 1.2. Hormones influenced by the gut microbiota and their metabolites.

Hormone	Description and function of hormone	Influence on metabolic function	Influence by gut microbiota	References
Peptide YY (PYY)	Enteroendocrine cell-derived hormone; may be involved in reducing appetite	Decreased PYY increase gut motility and transit rate; increase energy harvest	Gnobiotic mice (GPR41-deficient) colonized with *Bacteroides thetaiotaomicron* and *Methanobrevibacter smithi* show decreased PYY	Woods, 2005; Samuel et al., 2008
Fast-induced adipose factor (Fiaf)	Angiopoietin-like protein 4; expressed primarily in adipose tissue and has endocrine function	Reduced Fiaf results in increased lipoprotein lipase, aiding in triglyceride storage	Fiaf can be selectively suppressed by gut microbiota	Mandard, 2005
AMP-activated protein kinase (AMPK)	Initiates fatty acid oxidation	Suppression of AMPK initiates fatty acid oxidation in peripheral tissues of muscle and liver	Microbiota can suppress the activity of AMPK	Tilg and Kaser, 2011
Glucagon-like peptide 1 (GLP-1)	Key hormone released from enteroendocrine L-cells; promotes satiety	Increase glycogen synthesis in muscle cells and promotes satiety	Increased by fermentation of dietary fiber by gut microbiota and butyrate production	Baggio and Drucker, 2007

Table 1.3. Composition of different dietary fibers and their impact on gut microbiota.

Dietary fiber	Types	Structure	Impact on gut microbiota	Other health benefits	References
Arabinoxylan (AX)	Water-extractable; water un-extractable; arabinoxylan oligosaccharide (AXOS)	(1→4)-linked β-D-xylopyranosyl residues substituted with α-L-arabinofuranosyl residues	AX from wheat increase clostridial cluster XIV , *Bacteroides*, *Prevotella* spp; AXOS are strongly bifodogenic	Anti-inflammatory and anti-obesity effects; lower postprandial responses in serum glucose, insulin and triglycerides (AXOS)	Van Craeyveld et al., 2008;Van den Abbeele et al., 2011
β-Glucan (mixed linkage)		Liner chain of (1→4)- and (1→3)-linked β-D-glucopyranosyl residues	Increase *C. histolyticum*, and to a lesser extent clostridial cluster IX and the *Bacteroides–Prevotella* and *Atopobium* groups; moderate increase in propionate and butyrate	Increase satiety; reduce blood serum cholesterol and blood glucose	Snart et al., 2006; Hughes et al. 2008; Mitsou et al., 2010b
Fructans	Inulin, oligofructose, fructooligosaccharides (FOS)	(2→1)-linked β-fructofuranosyl residues with a terminal α-D-glucopyranosyl residue	Stimulation of *Bifidobacteria* growth	Increase calcium absorption; influence aspects of immune response in infants; therapeutic benefits in animal models of colitis	Kelly, 2008
Resistant starch	RS1 (physically inaccessible), RS2 (native granular), RS3 (retrograded), RS4 (chemically modified)	Linear or branched chain of (1→4)- and (1→6)-linked α-D-glucopyranosyl residues	High butyrate production; reduce production of toxic metabolites; RS-2 increase the phylotypes related to *Ruminococcus bromii*; RS-3 increased *Lactobacillus*; RS-4 increase *Actinobacteria* and *Bacteroidetes*, while decreasing *Firmicutes*	RS maintain blood glucose	Behall, 2006; Abell et al., 2008; Siew-Wai et al., 2010

Table 1.3 (continued). Composition of different dietary fibers and their impact on gut microbiota.

Type	Types	Structure	Impact on gut microbiota	Other health benefits	References
Glactooligosac-crides (GOS)	Human milk oligosaccharides (HMOs) and synthesized GOS	Synthesized GOS: A chain of galactose units containing (1→4), (1→3), and (1→6)-linkages with terminal glucose unit; HMOs: sialyic acid, fucose, 2-amino-glucuronic acid, glucose, and galactose with complicated linkages	Increased *Bifidobacteria* and *Lactobacilli*; decreased *Bacteroides*	Increased mineral absorption	Ben et al., 2008
Guar gum		A linear chain of (1→4)-linked mannose backbone and (1→6)-linked galactose branch chain	Increase in fecal *Bifidobacteria* compared with pectin	Increase satiety and delay gastric emptying	Noack et al., 1998

Table 1.4. Selected composition of different whole grains.

Grain	Dietary fiber composition (% dry basis)				Phenolic acids (mg/g)	Notes	References
	Arabinoxylan[a]	β-Glucan	Cellulose	Total			
Wheat	6-7 (25)	1	1.6-2.4	14.6	1.3	Highest betain content	Kulp and Ponte, 2000
Barley (hulless)	3-7 (14)	3-11	0.7-1.3	19.3-22.6	0.45-1.3	High in β-glucan	Delcour and Hoseney, 2010
Rye	6.5-12.2 (40)	1.9-2.9	1.3-1.9	14.6	1.4	Generally highest in WE-AX among whole grains	Kulp and Ponte, 2000
Oats (hulless)	1.2-2.9 (25)	4-6	0.8-2.0	9.6	0.47	Highest oil content; high in β-glucan; contains avenanthramides	Kulp and Ponte, 2000; Dykes and Rooney, 2007.
Corn	1-2	-[b]	1.9-2.5	13.4	0.60	Arabinoxylan contains more oligosaccharide branches compared with other whole grains	Delcour and Hoseney, 2010
Brown rice	2.64 (2.3)	0.11	1.4	3.9	0.20-0.38	High in phytosterols like oryzanol	Kulp and Ponte, 2000; Biliaderis and Izydorczyk, 2007
Sorghum (red)	1.8 (4.4)	0.9-3.15	2.7	10.1	0.39-0.75	Some cultivars contain condensed tannins	Bach et al., 1988;Kulp and Ponte, 2000; Delcour and Hoseney, 2010
Pearl millet	2-3	-	3	8.5	0.61-3.9	Highest calcium content among all cereal	Malathi and Devegowda, 2001; Falasca, 2009; Delcour and Hoseney, 2010; (Dendy, 1995)

[a] Parenthetic numbers indicate the approximate percentage of total arabinoxylan than is water-extractable; the remainder is water-unextractable.
[b] the component is not reported.

CHAPTER 2. IMPACT OF SELECTED DIETARY FIBERS ON GUT MICROBIOTA SHORT CHAIN FATTY ACID PRODUCTION AND CARBOHYDRATE UTILIZATION

2.1. Abstract

Aberrant gut microbiota have been associated with many metabolic diseases. Dietary fibers are an important promoter of differences in gut bacterial diversity. Few studies have assessed the impact of specific dietary fibers on gut microbial communities. Six dietary fibers [pectin, guar gum, inulin, arabinoxylan, β-glucan, and resistant starch type 2 (RS-2)] were subject to *in vitro* digestion and fermentation using fecal samples collected from 15 individuals. Pyrosequencing was used to assess the impact of dietary fibers on the gut microbiota community. Short/branched chain fatty acids and carbohydrate utilization were correlated with levels of bacterial taxa. RS-2 caused the most dynamic change of the whole microbiota community. *Bifidobacterium* increased almost 10-fold on pectin substrate compared with the control. A reduction in *Clostridiales* was detected on all dietary fiber substrates. *Ruminococcus* significantly increased on all substrates expect RS-2 and pectin. *Bacteroides* exhibited positive correlation with propionate ($r=0.52$, $p<0.01$), while *Ruminococcaceae* and *Faecalibacterium* displayed positive correlations with butyrate production ($r=0.39, 0.54$, $p<0.01$). A negative correlation was detected between inulin utilization and *Subdoligranulum* ($r=-0.74$, $p=0.004$) while a strong positive relationship was shown between β-glucan utilization and *Firmicutes* ($r=0.73$, $p=0.0019$). Fecal samples were also divided into two groups based on whether the donor was normal weight [body mass index (BMI) <25; n=7] or obese (BMI>30; n=8). *Incertae Sedis XIV* ($p<0.0001$), *Lachnospiraceae* ($p<0.04$), and *Ruminococcus* ($p<0.0001$) were significantly reduced in the obese compared with the normal weight group. Obese and normal weight microbiota responded in different fashions on each dietary fiber except RS-2, leading to different shifts in certain taxa.

2.2. Introduction

The human gut harbors a complex microbial community, referred to as the gut microbiota. There are at least 10^{14} microorganisms in the human colon belonging to over 1000 species (19). The genome size of the gut microbiota exceeds that of human genome by 100-fold, providing many metabolic functions that cannot be achieve alone (72). The gut microbiota has been linked to immune system development, protection from pathogens, and metabolic processes (66).

The importance of maintaining a "healthy" gut microbiota has been recognized. Aberrations in the gut microbiota, or dysbiosis, may lead to many metabolic diseases such as colon cancer, inflammatory bowel disease, type 2 diabetes, and obesity (44, 53). However, the underlying roles and functionality of specific bacteria relative to disease remain unclear (23).

Numerous studies (18, 42, 63) have shown that the diet is an important and fundamental promoter of differences in gut bacterial composition and diversity. One of the most promising means of manipulating the composition of the gut microbiota is with dietary fibers, which are the major source of energy for the gut microbiota. Because dietary fibers are a broad class of compounds with varying health benefits, certain dietary fibers can elicit differing effects on the gut microbiota. For instance, water extractable arabinoxylan (WE-AX), the most abundant non-digestible carbohydrates present in cereal grains, can be primarily fermented in the transverse colon and induce an increase in clostridial cluster XIV, *Bacteroides-Prevotella* spp., and *Bifidobacteria* accompanied by reduced circulating inflammatory markers, body weight, and hepatic cholesterol in mice (6). On the other hand, fructan generally supports an increase in *Bifidobacteria*, with numerous studies demonstrating its positive effects on obesity (49), diabetes (10), cancer (67), and other complications (38). Fermentation of resistant starch (RS) results in high production of butyrate (40) and RS4 (chemically modified starch) but not RS2 (high amylose starch) leads to increases in *Actinobacteria* and *Bacteroidetes* accompanied by decreases in *Firmicutes* (1).

Few studies have been performed to assess the impact of specific dietary fibers on gut microbial communities, especially using methodologies that amplify all bacteria in the community rather than pre-determined groups. Therefore, in the present study, pyrosequencing was used to determine shifts in the gut microbiota upon *in vitro* fermentation with different dietary fibers. The major metabolites [(short chain fatty acids (SCFA) and branched chain fatty acids (BCFA)] and carbohydrate utilization of bacteria were quantified and correlated with abundance of specific gut bacterial taxa to elucidate specific bacterial populations that play a role in the fermentation. Furthermore, because of potential differences in the gut microbiota between obese and normal weight individuals (41, 71), we also characterized differences in how the fecal microbiota from these two groups of people respond to each dietary fiber.

2.3. Materials and methods

2.3.1. Fecal samples

Twenty fecal samples were obtained from Rush University Medical Center (Table 2.1). Donors were identified from the university's patient database as having no known gastrointestinal disorders and having avoided antibiotics for 3 months prior to the study. Fecal samples were collected through a specimen hat inserted under the toilet seat, and then immediately transferred to gas-tight bags containing an Anaerocult C strip (BD GasPak Anaerobe Pouch system with indicator, NJ, USA), which created an anaerobic environment. Stools were stored at -80 °C until collection was complete, and then shipped overnight to the University of Nebraska-Lincoln on dry ice where they were further stored (-80 °C) until fermentation experiments were performed. Collection of these samples was approved by Rush University's Institutional Review Board (#10062307-IRB02).

Five fecal samples were excluded from the study. This is because with 15 donors we had 120 samples {[15 donors*(6 dietary fibers+1 control)] +15 fecal samples}, which was the maximum that could be accommodated in 1 pyrosequencing run and still give sufficient depth to

the data. Because we anticipated not only analyzing the differences among dietary fiber treatments, but also between obese and normal weight individuals, we excluded the three fecal samples from donors that were in the overweight category [body mass index (BMI) between 25 and 30] and the two from donors with the lowest BMIs in the obese category (BMI=31.4 and 31.7). This provided us with 7 fecal samples from normal weight individuals and 8 fecal samples from obese individuals with the greatest difference in BMI between the two groups.

2.3.2. Dietary fibers

Guar gum and pectin were obtained from TIC Gums (White Marsh, MD, USA). Organic agave inulin was provided by CIRANDA Innovative Organic Ingredients (Hudson, WI, USA). RS-2 (70% high amylose corn starch) was from Cargill, Inc. (Cedar Rapids, IA, USA). β-glucan from oats was extracted and purified as described (30), except the β-glucan was precipitated by adding 3 parts ethanol (by volume) rather than ammonium sulfate. AX was extracted as described (59), except arabinoxylan was bleached with hydrogen peroxide after centrifuging and removing solids rather than before.

2.3.3. *In vitro* digestion of resistant starch

Because the RS-2 preparation contained some digestible starch, this sample was subjected to *in vitro* digestion prior to fermentation. The digestion process consisted of a simulated gastric digestion followed by a small intestinal phase following Mishra and Monro (50) with some modifications. RS-2 (25 g) was boiled for 20 min with 300 ml distilled water in a 500 ml beaker inside of another larger beaker that was filled with boiling water. After cooling to room temperature, ~8 ml of 1M HCl were added to the sample to reduce the pH to 2.5. Ten ml of 10% (w/v) pepsin (P-700, Sigma, St. Louis, MO USA), dissolved in 0.05M HCl, was added and the mixture placed on an orbital shaker (150 rpm) at 37 ℃ for 30 min to achieve the gastric phase. The small intestinal phase was initiated with the addition of 50 ml 0.1 M sodium maleate buffer (pH=6, containing 1 mM $CaCl_2$) and ~20 ml of 1 M $NaHCO_3$ to bring the pH to 6.9. Fifty ml of

12.5% (w/v) pancreatin (P-7545, Sigma), dissolved in sodium maleate buffer, and 2 ml of amyloglucosidase (3260 U/ml; Magazyme) were then added and samples were incubated in a shaking water bath at 37 °C for 6 h. Digested contents were then poured into dialysis tubing (molecular weight cut off 12,000-14,000) and dialyzed for 3 d against distilled water with changing of the water every 12 h. The retentate was then frozen (-20 ℃) overnight and then freeze dried. The freeze dried, dialyzed material after digestion was analyzed for total starch and carbohydrate as described below.

2.3.4. *In vitro* fermentation

Dietary fiber samples and RS-2 after *in vitro* digestion containing 40 mg of total carbohydrate (sum of neutral sugar and uronic acid residues) were suspended in sterile nutrient basal medium (64) to a final concentration of 1% (w/v) and then hydrated overnight at 4 °C. Fecal samples were then taken from the freezer and defrosted in an anaerobic hood (Bactron IV, Sheldon manufacturing, Cornelius, OR USA) containing 5% H_2, 5% CO_2, and 90% N_2. Defrosted samples were weighed and mixed with sterile phosphate buffered saline at 10 % (w/v) (64); blended for 30 s using a kitchen blender (2774 heritage series, Sunbeam Company, Boca Raton, FL USA), and then filtered through 4 layers of cheesecloth. Tubes were then inoculated with 0.4 ml of fecal slurry, capped, placed at a 45° angle, and incubated at 37 °C with shaking (140 rpm). A sample containing only basal medium and fecal suspension was included as a control and a portion of fecal slurry was retained for a zero time reading. Samples were taken at 12 h by plunging in an ice bath and then freezing (-80 °C) until analysis. Prior to taking samples, the volume of gas produced by the bacteria was measured by inserting a lubricated glass syringe with needle through a septum in the cap of the tube.

2.3.5. Total starch and total carbohydrate assay

Total starch of RS-2 after *in vitro* digestion was performed using a kit (K-TSTA, Megazyme, Bray, Ireland) to check whether starch was digested during the *in vitro* digestion

process. Total carbohydrate as neural sugars plus uronic acids was determined in all the dietary fiber samples except inulin according to AACC International method 32-25 (29) with some modifications: 1) the sample size was decreased by half and regents used were decreased proportionally, 2) five μL of 2-octanol was added before the reduction step to minimize loss of ammonium hydroxide (Englyst and Cummings 1984), and 3) reduction time was increased to 1.5 h.. Inulin could not be determined in this way, since a significant portion of the fructose was degraded during acid hydrolysis. Thus, inulin content was measured using an enzymatic fructan kit (K-FRUC, Megazyme, Bray, Ireland). For β-glucan, a mixed-linkage β-glucan kit (K-BGLU, Megazyme, Bray, Ireland) was used for quantification of β-glucan specifically (total neutral sugars and uronic acids were also quantified in this sample).

Dietary fiber utilization was determined by analyzing total neutral sugars and uronic acids, or content of inulin and β-glucan as described above, after *in vitro* fermentation on collected fecal slurries and subtracting from the content at the beginning of fermentation.

2.3.6. Short chain fatty acid assay

SCFA were analyzed based on Campbell et al. (8). One ml of sample was taken from -80 °C freezer and thawed at room temperature. Metaphosphoric acid (0.25 ml 5%, w/v) containing 5-10 mM 4-methylvaleric acid (A15405, Alfa Aesar, MA, USA) was added into the sample tube as an internal standard. Then the tubes were vortex mixed and centrifuged for 20 min at 16,000 rpm. Four μL were then injected onto a gas chromatograph (Clarus 580, PerkinElmer, Waltham, MA USA) and SCFA were separated on a capillary column (Elite-FFAP, 15 m × 0.25 mm inner diameter × 0.25 μm film thickness, PerkinElmer) and detected with a flame ionization detector. Quantification was accomplished by calculating response factors for each SCFA relative to 4-methylvarleric acid using injections of pure standards.

2.3.7. Composition analysis of the fecal microbiota by pyrosequencing

2.3.7.1. DNA extraction

Fermentation slurries (1 ml) were thawed and the cells were recovered by centrifugation (10,000g for 5 min at room temperature). The pellet was resuspended in 0.6 ml stool lysis buffer (Buffer ASL, QIAGEN, Maryland, USA) and transferred to a tube containing 300 mg of zirconium beads (0.1 mm. BioSpec Products). DNA extraction and purification were then processed by the University of Nebraksa-Lincoln's Core for Applied Genomics and Ecology (CAGE) with a Qiagen BioSprint 96 using the BioSprint 96 One-For–All Vet kit and Protocol (Purification of Viral Nucleic Acids and Bacterial DNA from Animal Tissue Homogenates, Swab Media, and Cell-Free Body Fluids).

2.3.7.2. Pyrosequencing and data analysis

Sequencing was performed by CAGE. The V1-V3 region of the 16S rRNA gene was amplified by PCR from DNA using primers adapted for the Roche-454 Titanium kit. A mixture (4:1) of the primers B-8FM (5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGAGAGTTTGATCMTGGCTCAG-3') and B-8FMBifido (5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGAGGGTTCGATTCTGGCTCAG-3'), were used as the forward primers. The primer A-518R (5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGBBBBBBBBBATTACCGCGGCTGCTGG-3') containing an 8-base barcode sequence (represented by Bs) was used as the reverse primer. The B-8FMBifido primer was used to account for the mismatches that bacteria of the genus *Bifidobacterium* have to the 8FM primer and avoid underrepresentation of this taxa. Equal amounts of the PCR products were combined and gel purified. Sequencing was performed from the A end primer with the 454/Roche A sequencing primer kit using a Roche Genome Sequencer GS-FLX following manufacturer's protocol for the Roche 454 GS FLX Titanium.

2.3.7.3. Pyrosequencing data processing pipelines

Sequencing process and analysis were based on the combination of QIIME (11) and Ribosomal Database Project (RDP) (13) pipelines. Sequences were then assigned to their respective samples via the barcode. Quality control of the sequences was performed with QIIME to remove sequences with the following attributes: 1) sequences longer than 550 bp or shorter than 300 bp; 2) sequences with one or more ambiguous nucleotides; 3) sequences with average quality score below 25 4) reads containing homopolymer runs larger than 6 bp; and 5) sequences with more than one mismatch to the primer or barcode. Bacterial 16S rRNA sequences were classified from the phylum to the genus levels using the Classifier tool (RDP). Local Blasts were performed using the standalone ncbi-blastn 2.2.25+ tool with 16S rRNA sequences of type strains of bacteria identified as core species of the human microbiome or relevant in carbohydrate fermentation as the query sequences (56) and a created local database containing the 16S rRNA sequences generated in our study. Operational Taxonomic Unit (OTU) picking with 97% similarity cutoff was performed with QIIME using default parameters. These data were used to calculate UniFrac beta-diversity distances and generate the corresponding principal component analysis (PCA) plots with QIIME.

2.4. Statistical analysis

Differences among dietary fiber treatments and UniFrac index were computed using one-way ANOVA in combination with Tukey's post-hoc tests using SAS software (version 9.2, SAS Institute, Cary, NC USA). Pearson's coefficients were computed for correlation analysis. Average carbohydrate utilization was determined using ANOVA followed by Fisher's protected least significant difference. T-test was performed to compare the difference in bacterial taxa in fecal samples and samples obtained after *in vitro* fermentation between obese and normal weight individuals on each dietary fiber. $P<0.05$ was used to consider statistical significance. Data were expressed as mean±standard error.

2.5. Results and discussion

2.5.1. Characteristics of dietary fiber used for *in vitro* fermentation

Carbohydrate compositions of the dietary fibers used in this study are shown in Table 2.2. Dietary fiber does not refer to a single compound, instead, contains a broad class of compounds with different chemical structures. Commercial pectin is a linear chain of 1, 4-linked α-D-galacturonic acid containing varying degrees of methylation (51). Guar gum is a polysaccharide composed of galactose residues 1,6- linked to a β-D-mannosyl backbone. The inulin used in our study was from agave, with an average DP of 14 (58). The alkali-solubilized AX used in this study consisted of a backbone of 1, 4-linked β-D-xylopryanosyl residues, substituted with oligosaccharide branches containing arabinose, galactose, glucuronic acid, and xylose (59). β-Glucan is composed of β-D-glucosyl units linked by 1, 3 and 1, 4 glycosidic bonds. RS-2 represents starch that is resistant to digestion of human small intestinal enzymes (61).

Prior to the *in vitro* fermentation process with human fecal microbiota, *in vitro* digestion, which imitated the digestion occurring in human gastrointestinal tract, was performed to remove digestible starch from the RS-2 preparation (about 20%; Table 2.2). Other purified dietary fibers were directly subjected to *in vitro* fermentation. The β-glucan content measured using an enzymatic colorimetric kit was slightly higher than the total glucan content using gas chromatography; these differences may due to the differences in methodology.

2.5.2. Bacterial taxa that were affected by dietary fiber treatments

Compared with *in vivo* studies, there are limitations to the *in vitro* experimental approach. *In vitro* fermentation does not exactly replicate the conditions that occur in the large intestine, resulting in blooming or diminishing of certain bacteria taxa. Nevertheless, this methodology is correlated well with dietary fiber fermentation *in vivo* and can provide insight into the fermentation profile of different carbohydrates (2). In an effort to separate the shifts in the gut microbiota profile resulting from the *in vitro* environment and the shifts brought on by the dietary

fiber treatment, a blank containing no carbohydrate substrate was included alongside the fermentations.

Weighted UniFrac indeces were used to construct PCA graphs comparing each dietary fiber treatment with the control containing no dietary fiber (Figure 2.1). Pectin and RS-2 led to a more dynamic shift in the bacterial community; other substrates resulted in extensive overlapping between the bacterial community of control and dietary fiber. Although 38.5% of the variation in composition could be explained by dietary fiber treatments, large individual difference in bacterial communities resulted in much overlapping in the PCA figures.

To further evaluate the shift of bacterial community, phylogenetic beta diversity using UniFrac was calculated (Figure 2.2), revealing the relative distance of bacterial communities between the original fecal samples and the communities after *in vitro* fermentation. Only RS-2 showed a significant impact on shifting the bacterial community compared with the control, although differences were observed among dietary fiber treatments.

Table 2.3 shows the shifts in specific bacterial taxa induced by different dietary fiber treatments. The most substantial change was in *Bifidobacterium*, which increased almost 10-fold on pectin compared with the control. Pectin oligosaccharides have been reported to have bifidogenic prebiotic properties (28), while research on commercial pectin is limited. Although the commercial pectin we used contained some free glucose, this carbohydrate is not known to have prebiotic properties and should not have affected the growth of *Bifidobacterium* (46). *Bifidobacterium* is thought to play an important role in the improvement of health. For instance, protection from enteric infection, suppression of pathogenic bacteria by lowing the intestinal pH through breaking down carbohydrates, stimulation of immune response, protecting gut barrier and so on (38). Also, suppressed *Bifidobacterium* has been shown to be associated with obesity (49).

Other substrates did not result in significant changes in *Bifidobacterium*. This was surprising, as inulin is usually considered a bifidogenic carbohydrate, and, although we did see a

numeric increase in *Bifidobacterium* on inulin, it did not reach significance. The DP of the inulin used in this study was fairly high compared with other studies and others have shown that the bifidogenicity of inulin-type fructans decreases as DP increases (36, 37). Also, others have shown that about 30% of the population are "non-responders" with respect to the bifidogenicity of inulin (35); perhaps the subjects in our study had a high percentage of "non-responders." Additionally, *Subdoligranulum* was enhanced in fermentation residues with high inulin utilization (discussed below). *Subdoligranulum* can produce bacteroicins that could have inhibited growth of *Bifidobacteria*.

Another significant change was in *Erysipelotrichaceae,* which significantly increased on all dietary fiber substrates, especially on β-glucan and inulin. Higher proportion of *Erysipelotrichaceae* was found with mice fed a high fat diet (21), while other studies indicated that four different lineages of this family respond differentially to high fat diets (78). Few studies have evaluated the association between this family and carbohydrate consumption (70).

On the order level, a reduction of *Clostridiales* was detected on all dietary fibers substrates, although this was only significant on RS-2 and pectin substrates. Propensity to high-fat induced obesity and gut inflammation has been linked to high proportions of this bacterial group (17). Furthermore, compared with the control, *Ruminococcus* was significantly higher on all substrates expect RS-2 and pectin. *Ruminococcus* has also been reported to favor weight loss in one study (62). This suggests dietary fibers may inhibit diet induced obesity by inducing a reduction in *Clostridiales* and increasing *Ruminococcus.*

All dietary treatments increased the proportion of *Actinobacteria*, though only pectin treatment reached significance. Among different dietary fibers, the increase in *Bacteroidetes* was significantly higher on AX than other treatments. *Dorea* was reduced on pectin and inulin substrates. Martínez et al (47) also reported a significant decrease of this genus upon resistant starch consumption *in vivo*. A 10-fold increase in *Collinsella* was detected on inulin substrate, a

genus that has been found less abundant in irritable bowel syndrome patients than in healthy subjects (34).

2.5.3. Associations between the gut microbiota and metabolites (SCFA and BCFA)

Major bacterial metabolites, SCFA and BCFA, were correlated with all bacterial taxa (Table 2.4). *Blautia* and *Bifidobacterium* showed a positive correlation with total SCFA production, while *Proteobacteria, Gammaproteobacteria, Enterobacteriales,* and *Eggerthella* displayed a negative relationship. Although SCFA can increase the energy harvest from dietary fiber, it only accounts for about 10% total energy harvest from food in humans (48). Furthermore, SCFAs contribute several beneficial effects on gut health, including nourishment of the colonic mucosa, pathogen resistance and inhibition of cholesterol synthesis (25). Furthermore, they play a role in regulation of hormones on energy metabolism. For instance, activation of GPR41 leads to up regulation of leptin and peptide YY, two impotent hormones that reduce the appetite (7, 33, 39, 52, 55).

The proportion of one phylum, *Bacteroidetes*, exhibited strong positive correlation with both the amount of propionate and propionate proportion. A similar correlation was observed in *Bacteroidia* (class), *Bacteroidales* (order) and *Bacteroides* (genus), indicating that *Bacteroides* (genus) had an impact on the propionate production. This finding is in accordance with previous research that *Bacteroides* are propionate producers through the succinate pathway (45). Propionate that is absorbed through the intestinal wall primarily acts as a precursor for gluconeogenesis, although some animal studies have suggested that it can help reduce hepatic cholesterol synthesis (12, 75). In addition, propionate has also been shown to decrease glucose-induced insulin secretion in isolated pancreatic islet cells of rats (76).

Butyrate was moderately correlated with the proportion of *Firmicutes, Clostridia, Incertae Sedis XIV, Blautia* and *Eubacterium,* while *Ruminococcaceae* and *Faecalibacterium* displayed a robust positive correlation with butyrate production. *Ruminococcaceae*, a member of

Clostridia, is one of the primary butyrate-producing bacteria found in human feces (45). Benus et al. (4) showed strong positive correlation between the numbers of *Faecalibacterium* and the production of butyrate. The preferred energy source of coloncytes, butyrate plays an important role in the control of the machinery regulation apoptosis and cellular proliferation, resulting in lowering the risk of colon cancer (9). Butyrate has also been proposed to ameliorate mucosal inflammation (9). *Faecalibacterium prausnitzii* has been identified as an anti-inflammatory commensal bacterium (68), which may be a result of butyrate production from this genus.

Because SCFAs are produced as a result of carbohydrate fermentation and BCFAs represent a good marker of undesirable protein fermentation, SCFA/BCFA could function as an indicator of the propensity of the gut microbiota toward carbohydrate fermentation or protein fermentation. Although no detrimental impacts have been proposed by BCFA, the presence of them are often accompanied with production of toxic metabolites such as ammonia (5, 60). *Ruminococcaceae* indicated a stronger positive association with SCFA to BCFA ratio. Thus *Ruminococcaceae* may either compete with some bacterial taxa that fermented protein or increase the fermentation of carbohydrates, thereby reducing fermentation of protein and production of BCFA.

2.5.4. Associations between the gut microbiota and carbohydrate utilization

In an effort to deepen our understanding of how dietary fibers are utilized by gut microbial communities, we assessed associations between the gut microbiota and carbohydrate utilization (Figure 2.3). The gut microbiota were most efficient in using pectin. Notably, the gut microbiota were more efficient at utilizing linear carbohydrate polymers [pectin, inulin, β-glucan, resistant starch (amylose)] compared with those with side chains (AX, guar gum). Branched regions in AX and guar gum may provide resistance to hydrolysis by bacterial enzymes, thus influencing the efficiency for bacterial usage.

Fermentation of dietary fibers may select for certain bacteria that are especially equipped to utilize that particular polymer (77). Thus, we correlated the carbohydrate utilization during fermentation with all the bacterial taxa (Table 2.5). *Bifidobacterium* was positively associated with the utilization of RS-2. Certain *Bifidobacterium* are amylolytic and efficient at utilizing starch after adhering to the starch granule (16, 57). A strong positive relationship was shown between β-glucan consumption and the proportion of *Firmicutes*. *Firmicutes* phylum accounts for up to 90% total bacteria in our gut, containing members of clostridial cluster IV, XIVa, and other groups with different functionality. Although few studies have been conducted to understand the mechanism by which bacteria in this phylum use dietary fibers substrates, *R. flavefaciens,* belonging to *Firmicutes,* possesses the ability to produce a wide range of enzymes, such as glycoside hydrolases (endo-1,4-β-glucanase), polysaccharide lyases and carbohydrate esterases that can be used to metabolize various dietary fiber polymers (32, 54).

Our analyses also revealed a robust negative relationship between the inulin utilization and *Subdoligranulum*. *Subdoligranulum* is phylogenetically a member of *Clostridium leptum* (27), and produces bacteriocins, which are protein toxins that suppress the growth of similar or closely related bacterial strains (20). Bacteriocins produced by this genus may have played a role in suppressing *Bifidobacteria* growth during inulin fermentation and explain the lack of a significant increase in this genus.

2.5.5. Dietary fiber, gut microbiota and obesity

Obesity is a major health concern in the US. Thus, we desired to determine differences in fecal samples and fermentation profiles on different dietary fibers among the fecal samples in our study.

2.5.5.1. Fecal gut microbiota composition from obese and normal weight individuals

As mentioned, the gut microbiota in obese individuals is likely different from normal weight individuals (15, 22, 43, 65, 79), although results are not conclusive. Thus, three million

quality-controlled, chimera-free 16S rRNA gene tags per sample were used to build the taxonomic profile of the microbial communities in fecal samples collected and the values were expressed as percent taxa abundance to the total number of sequences. At the phylum level, the microbiota in both groups were dominated by *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia* (Table 2.6), which is similar to previous reports (24, 26). No significant differences were detected between obese and normal weight groups, in accordance with Duncan et al (18). A non-significant trend toward a reduction in *Firmicutes* and more *Bacteroidetes* and *Actinobacteria* in obese compared with normal weight was noted. Perhaps with more individuals the results would have been significant in accordance with Ley et al (42), who reported that obese individuals display a reduced *Bacteroidetes* to *Firmicutes* ratio compared with lean individuals. Zhang and Schwiertz et al, (65, 79) on the other hand, reported more *Bacteroidetes* in obese individuals. Given that individuals harbor gut microbial communities whose relative proportions of predominant microbial groups are tremendously varied (3), and *Firmicutes* and *Bacteroidetes* contain over 250 genera with different properties (14), it is not surprising that our results and those found in the literature are so varied.

At the family level, *Incertae Sedis XIV* were approximately three-fold lower in the obese group compared with the normal weight group. Reduced *Incertae Sedis XIV* has been reported in patients with pouchitis, a type of inflammatory bowel disease (69), though the relationship with obesity remains unknown. *Lachnospiraceae*, a butyrate producer, were lower in the obese group compared with the normal weight group. It has been reported that *Lachnospiraceae* was less abundant in colon cancer patients (73), which may be explained by the beneficial effects of butyrate.

At the genus level, *Ruminococcus* were significantly reduced in the obese group compared with the normal weight group. *R. flavefaciens*, a subgroup of *Ruminococcus*, has been observed abundant in normal individuals compared with obese (65). In addition, *R. bromii* are

one of the major starch degraders in the colon, producing acetate, butyrate and propionate, which has been linked to some health benefits (31, 74).

2.5.5.2. Difference between obese and normal weight group after in vitro fermentation within each dietary fiber

We compared the abundance of each bacterial taxa within the gut microbiota community after *in vitro* fermentation between the obese and normal weight groups within each dietary fiber (Table 2.7). We found that the obese and normal microbiota responded in different fashions within the dietary fiber, except when RS-2 was used as substrate. For instance, inulin resulted in about 9-fold increase of *Bacilli* in normal weight group compared with the obese group. The different initial composition between obese and normal weight gut microbiota is likely a contributing factor to different fermentation responses on the same substrate. Different response between obese and normal weight microbiota could also be due to differences in metabolic activity.

2.6. Conclusion

Our results showed that RS-2 caused the most dynamic change of the whole microbiota community while *Bifidobacterium* increased almost 10-fold on pectin. *Ruminococcaceae* and *Faecalibacterium* displayed a robust positive correlation with butyrate production. We also found a strong positive relationship was shown between β-glucan consumption and the proportion of *Firmicutes*, while a robust negative relationship between the inulin utilization and *Subdoligranulum.*

We also found small but significant differences in the gut microbiota between obese and normal weight individuals, namely *Incertae Sedis XIV* (family), *Lachnospiraceae* (family), and *Ruminococcus* (genus) were reduced in the obese microbiota. These differences resulted in slightly different fermentation profiles when exposed to the same dietary fiber. Given the impact of the microbiota of health, more research is needed to explore the association of gut microbiota

and dietary fiber degrading activities, so as to influence the gut microbiota to the most desirable composition for health.

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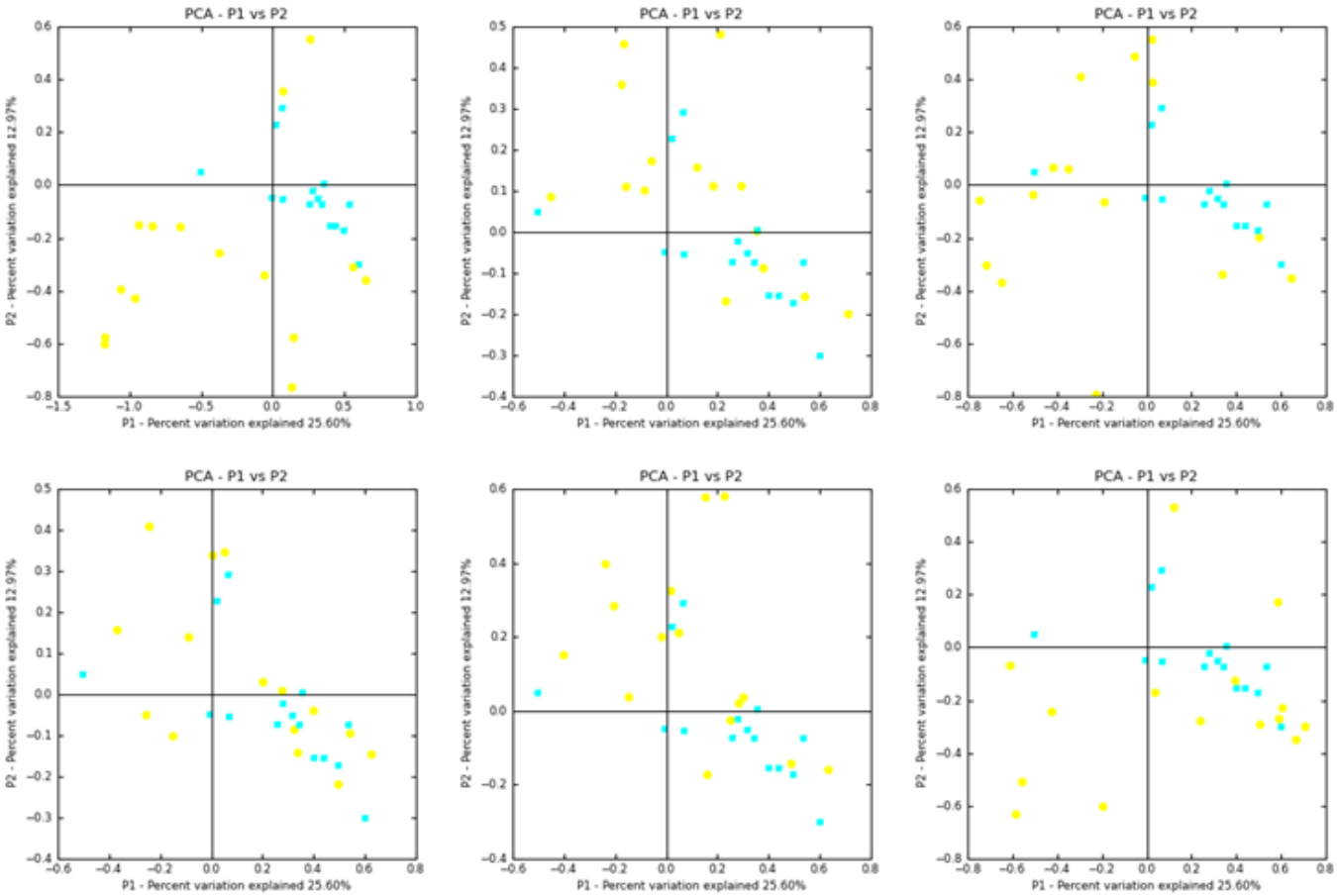

Figure 2.1.Fecal bacterial communities clustered using principal coordinate analysis of the UniFrac matrix; control (no dietary fiber during fermentation) is in blue and dietary fibers are in yellow; clockwise from top left: pectin, guar gum, inulin, resistant starch type 2, β-glucan, and arabinoxylan.

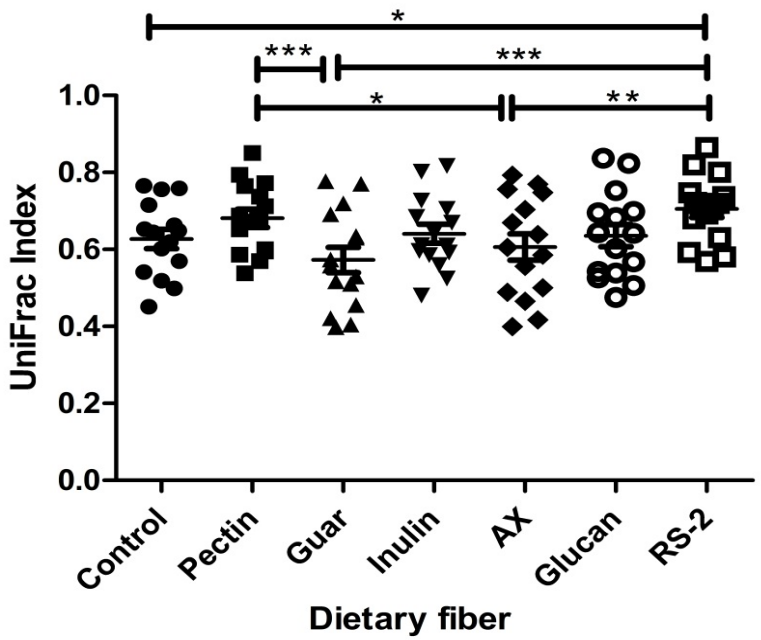

Figure 2.2. Shift in the gut microbiota based on UniFrac metrics comparing initial fecal sample with final microbiota composition after 12 h of *in vitro* fermentation for each dietary fiber treatment and control containing no carbohydrate; n=15; *p<0.05, **p<0.01, ***p<0.0001.

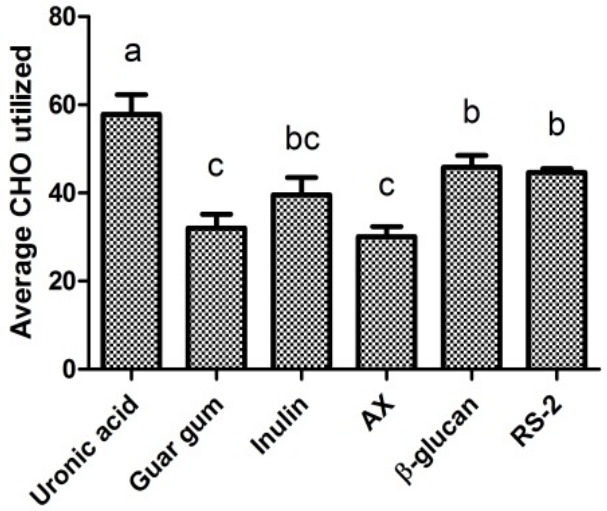

Figure 2.3. Average carbohydrate (CHO) utilization (%) of each dietary fiber during *in vitro* fermentation; error bars show standard error from different fecal samples; bars with different letters are significantly different (n=15; $p<0.05$); AX, arabinoxylan; RS-2, resistant starch type 2.

Table 2.1. Fecal sample donor information

Patient #	BMI[a]	Assigned group	Age	Gender	Race	Bowel movements per week
19	18.4	Normal weight	46	Male	Caucasian	10
20	20.7	Normal weight	24	Female	Caucasian	6
3	21.8	Normal weight	32	Female	Caucasian	2
8	22.1	Normal weight	31	Male	AA[b]	17
14	22.1	Normal weight	51	Female	AA	6
2	24.1	Normal weight	44	Female	AA	2
29	24.1	Normal weight	31	Female	AA	3
18	25.1	Overweight	56	Male	AA	15
7	25.3	Overweight	56	Female	AA	9
10	26.1	Overweight	23	Male	AA	7
9	31.4	Obese	54	Male	AA	3
15	31.7	Obese	23	Female	AA	12
21	33.2	Obese	51	Female	Hispanic	4
5	36.7	Obese	50	Female	AA	7
4	36.9	Obese	44	Male	AA	6
6	40.1	Obese	52	Male	AA	4
16	40.6	Obese	31	Female	Hispanic	4
17	40.9	Obese	46	Female	Caucasian	13
11	42.8	Obese	29	Female	AA	5
23	45.4	Obese	33	Female	AA	6

[a]BMI: Body mass index
[b]AA: African American

Table 2.2. Composition of dietary fibers used for *in vitro* fermentation. [a]

	% dry basis					
Constituent	Pectin	Guar gum	Inulin	AX[b]	β-glucan	RS-2[c]
Arabinose	1.26±0.05	2.03±0.08	NA[d]	22.73±0.31	1.81±0.02	0.34±0.06
Xylose	0.18±0.06	0.49±0.01	NA	43.92±0.2	1.98±0.11	0.29±0.03
Manose	0.32±0.32	46.60±0.89	NA	0.13±0.05	1.09±0.21	0.83±0.002
Galactose	2.22±0.24	28.22±0.53	NA	6.48±0.14	0.32±0.28	0.35±0.01
Glucose	38.47±0.86	2.48±0.13	NA	4.66±0.24	73.92±0.04	59.21±0.43
Uronic acid	35.41±0.22	NA	NA	4.21±0.02	ND[e]	ND
Inulin	NA	NA	88.21±0.00	NA	NA	NA
β-glucan	NA	NA	NA	NA	76.64±0.05	NA

[a] Mean values ± SE; n=2. Neutral sugars and uronic acids expressed as they would occur in a polysaccharide (0.9*weight of hexose or 0.88*weight of pentose); [b]AX: Arabinoxylan; RS-2: Resistant starch type 2. Starch digested during in *vitro* digestion process was 19.80±0.31%. Prior to *in vitro* digestion, RS2 contained 80.20±0.31% starch; [d]NA = not analyzed; [e]ND = not detected.

Table 2.3. Abundance of bacterial taxa that were affected by dietary fiber treatments.[a,b]

	Control	Pectin	Guar gum	Inulin	AX	β-glucan	RS-2
Phylum							
Actinobacteria	4.99±1.75^{b}	39.03±7.84^{a}	5.07±1.24^{b}	20.63±5.11^{b}	4.99±1.54^{b}	6.29±1.56^{b}	15.61±5.15^{b}
Bacteroidetes	8.57±2.01^{abc}	0.87±0.23^{c}	12.43±2.86^{ab}	1.72±0.53^{c}	16.24±3.24^{a}	5.19±1.60^{bc}	5.90±1.90^{bc}
Class/subclass							
Actinobacteria	4.99±1.75^{b}	39.03±7.84^{a}	5.07±1.24^{b}	20.63±5.11^{b}	6.29±1.56^{b}	4.28±1.54^{b}	15.61±5.15^{b}
Bacteroidia	8.56±2.01^{ab}	0.87±0.22^{c}	12.41±2.85^{ab}	1.71±0.53^{c}	16.23±3.64^{a}	5.18±1.60^{bc}	5.90±1.90^{bc}
Actinobacteridae	3.004±0.96^{b}	29.94±7.94^{a}	3.56±0.97^{b}	9.72±3.03^{b}	5.03±1.45^{b}	1.35±0.36^{b}	10.56±4.72^{b}
Coriobacteridae	1.98±1.03^{b}	9.03±2.86^{ab}	1.49±0.57^{b}	10.88±3.82^{a}	1.25±0.48^{b}	2.93±1.32^{ab}	5.01±2.25^{ab}
Order/suborder							
Bacteroidales	8.56±2.01^{abc}	0.87±0.22^{c}	12.42±2.85^{ab}	1.71±0.53^{c}	16.23±3.64^{a}	5.18±1.60^{bc}	5.90±1.89^{bc}
Bifidobacteriales	2.9±0.95^{b}	29.91±7.94^{a}	3.58±0.97^{b}	9.70±3.04^{b}	5.02±1.50^{b}	1.34±0.36^{b}	10.54±4.72^{b}
Clostridiales	44.62±4.83^{a}	19.46±4.31^{b}	42.08±4.96^{a}	33.99±7.73^{ab}	42.84±5.30^{a}	40.06±4.64^{ab}	20.61±3.56^{b}
Coriobacteriales	1.98±1.03^{ab}	9.03±2.87^{ab}	1.49±0.57^{b}	10.88±3.38^{a}	1.25±0.48^{b}	2.93±1.32^{ab}	5.01±2.25^{ab}
Erysipelotrichales	2.28±0.82^{b}	7.22±3.13^{ab}	6.49±1.74^{ab}	19.60±6.46^{a}	3.52±0.88^{b}	20.73±4.59^{a}	6.58±1.75^{ab}
Actinomycineae	0.01±0.003^{a}	0.001±0.001^{b}	0.001±0.001^{ab}	0.001±0.001^{b}	0.001±0.001^{b}	ND^{b}	0.005±0.005^{ab}
Coriobacterineae	1.98±1.03^{ab}	9.03±2.87^{ab}	1.49±0.57^{b}	10.88±0.38^{a}	1.25±0.48^{b}	2.93±1.32^{ab}	5.01±2.25^{ab}
Family							
Actinomycetaceae	0.01±0.003^{a}	0.001±0.001^{b}	0.001±0.001^{ab}	0.001±0.001^{b}	0.001±0.001^{b}	ND^{b}	0.005±0.004^{ab}
Bacteroidaceae	7.00±1.86^{abc}	0.69±0.19^{c}	11.04±2.74^{ab}	1.36±0.48^{c}	15.33±3.65^{a}	4.58±1.45^{bc}	5.35±1.85^{bc}
Bifidobacteriaceae	2.96±0.95^{b}	29.91±7.94^{a}	3.55±0.97^{b}	9.70±3.03^{b}	5.02±1.44^{b}	1.34±0.35^{b}	10.54±4.72^{b}
Coriobacteriaceae	1.98±1.03^{ab}	9.03±2.87^{ab}	1.49±0.57^{b}	10.88±3.38^{a}	1.25±0.48^{b}	2.93±1.32^{ab}	5.01±2.25^{ab}
Erysipelotrichaceae	2.28±0.82^{c}	7.22±3.13^{ab}	6.49±1.74^{ab}	19.60±6.46^{a}	3.52±0.88^{b}	20.73±4.59^{a}	6.58±1.79^{ab}
Incertae Sedis XI	0.057±0.029^{a}	ND^{b}	0.008±0.004^{b}	ND^{b}	0.004±0.002^{b}	0.013±0.010^{ab}	0.0008±0.0001^{b}

Table 2.3 (continued). Abundance of bacterial taxa that were affected by dietary fiber treatments.[a,b]

	Control	Pectin	Guar gum	Inulin	AX	β-glucan	RS-2
Incertae Sedis XIV	2.44±0.44^{b}	3.92±0.97^{ab}	1.93±0.19^{b}	4.73±2.18^{ab}	4.77±0.74^{ab}	10.24±3.77^{ab}	0.90±0.22^{b}
Porphyromonadaceae	1.43±0.40^{a}	0.13±0.06^{b}	1.01±0.3^{ab}	0.09±0.03^{b}	0.72±0.15^{ab}	0.49±0.19^{ab}	0.43±0.40^{ab}
Genus							
Anaerostipes	0.024±0.01^{a}	ND^{b}	0.009±0.004^{ab}	0.01±0.004^{ab}	0.003±0.002^{ab}	0.006±0.003^{ab}	0.01±0.006^{ab}
Anaerovorax	0.056±0.028^{a}	0.033±0.018^{ab}	0.009±0.004^{ab}	0.007±0.003^{ab}	0.02±0.005^{ab}	0.006±0.003^{ab}	0.002±0.002^{b}
Bacteroides	7±1.86^{abc}	0.69±0.19^{c}	11.04±2.74^{ab}	1.36±0.48^{c}	15.33±3.65^{a}	4.58±1.45^{bc}	5.35±1.85^{bc}
Bifidobacterium	2.88±0.92^{b}	29.26±7.79^{a}	3.88±0.95^{b}	9.49±2.98^{b}	4.94±1.42^{b}	1.31±0.35^{b}	10.30±4.62^{b}
Blautia	2.43±0.44^{c}	3.9±0.97^{ab}	1.92±0.19^{c}	4.72±2.18^{ab}	4.75±0.74^{ab}	10.23±3.77^{a}	0.90±0.22^{c}
Butyricicoccus	0.23±0.06^{ab}	0.06±0.03^{b}	0.53±0.16^{a}	0.10±0.04^{b}	0.13±0.032^{b}	0.12±0.045^{b}	0.05±0.03^{b}
Collinsella	1.72±0.1^{b}	8.88±2.84^{ab}	2.16±0.56^{b}	10.77±3.38^{a}	1.13±0.47^{b}	2.85±1.31^{ab}	4.92±2.24^{ab}
Coprobacillus	0.94±0.61^{b}	2.28±1.1^{b}	3.73±1.77^{ab}	5.24±3.53^{ab}	1.92±0.63^{b}	12.73±4.30^{a}	2.37±1.29^{b}
Dorea	8.47±2.49^{a}	0.50±0.13^{b}	4.43±1.10^{ab}	1.01±0.35^{b}	5.03±1.29^{ab}	3.01±1.29^{ab}	4.07±1.39^{ab}
Eggerthella	0.14±0.038^{a}	0.062±0.003^{ab}	0.042±0.010^{b}	0.035±0.019^{b}	0.033±0.010^{b}	0.033±0.011^{b}	0.038±0.015^{b}
Holdemania	0.03±0.009^{a}	0.008±0.006^{ab}	0.004±0.002^{b}	0.002±0.001^{b}	0.011±0.005^{ab}	0.001±0.001^{b}	0.002±0.002^{b}
Parabacteroides	1.42±0.40^{a}	1.13±0.05^{b}	1.0±9.36^{ab}	0.086±0.03^{b}	0.71±0.15^{ab}	0.49±0.19^{ab}	0.43±0.19^{ab}
Peptoniphilus	0.02±0.013^{a}	ND^{b}	ND^{b}	ND^{b}	0.002±0.001^{b}	0.003±0.002^{b}	ND^{b}
Roseburia	0.18±0.04^{b}	0.17±0.07^{b}	3.16±1.19^{a}	0.44±0.21^{b}	0.83±0.32^{b}	0.99±0.43^{ab}	0.95±0.50^{ab}
Others							
Unclassified Bacteroidales	0.096±0.022^{abc}	0.016±0.006^{bc}	0.11±0.027^{a}	0.022±0.008^{bc}	0.20±0.022^{ab}	0.057±0.017^{bc}	0.062±0.017^{abc}
unclassified Firmicutes	1.07±0.28^{ab}	0.46±0.21^{b}	1.20±0.46^{ab}	1.32±0.80^{ab}	0.98±0.21^{ab}	3.40±1.28^{a}	0.52±0.24^{b}
unclassified Ruminococcaceae	1.02±0.29^{b}	0.66±0.29^{b}	5.84±2.56^{a}	0.44±0.16^{b}	0.56±0.16^{b}	1.4±0.55^{b}	0.24±0.086^{b}
unclassified Actinobacteria	0.004±0.002^{b}	0.063±0.015^{a}	0.013±0.007^{b}	0.032±0.012^{ab}	0.009±0.004^{b}	0.003±0.002^{b}	0.031±0.017^{ab}
unclassified Bifidobacteriaceae	0.08±0.027^{b}	0.65±0.16^{a}	0.07±0.02^{b}	0.21±0.06^{b}	0.08±0.027^{b}	0.032±0.01^{b}	0.24±0.11^{b}
unclassified Incertae Sedis XIII	0.049±0.019^{a}	0.007±0.003^{b}	0.007±0.002^{b}	0.007±0.003^{b}	0.011±0.005^{b}	0.007±0.004^{b}	0.016±0.007^{b}

[a]Values are reported as mean ± SE (n=15); means in the same row followed by the same letter are not significantly different ($p<0.05$); [b]Bacterial taxa that did not have any statistical significance between different dietary treatments were removed.

Table 2.4. Correlation between bacterial metabolites and bacterial populations.[a b]

	Total SCFA	Butyrate	Propionate	Butyrate/SCFA	Propionate/SCFA	SCFA/BCFA[c]
Phylum						
Acidobacteria						0.23*
Actinobacteria	0.24*				-0.37**	
Bacteroidetes			**0.58****		**0.59****	0.27*
Firmicutes		0.29**	0.31**			
Fusobacteria					0.42**	
Proteobacteria	-0.25*	-0.33**	-0.27**		0.23**	
Verrucomicrobia						0.22*
Class/subclass						
Actinobacteria			-0.23*		-0.37**	
Bacteroidia			**0.58****		**0.59****	0.27**
Clostridia		0.24*				
Gammaproteobacteria	-0.25*	-0.32**		-0.28**	0.22*	
Actinobacteridae	0.32**				-0.31**	
Coriobacteridae			-0.21*		-0.25*	
Peptococcaceae					0.26*	0.2*
Actinomycineae	-0.28**					
Coriobacterineae					-0.25*	
Order						
Actinomycetales	-0.26**					
Aeromonadales					0.3**	
Bacteroidales			**0.59****		**0.59****	-0.27**
Bifidobacteriales	0.32				-0.31**	
Clostridiales		0.24*				
Coriobacteriales			-0.21*		-0.25*	

Table 2.4 (continued). Correlation between bacterial metabolites and bacterial populations.[a b]

	Total SCFA	Butyrate	Propionate	Butyrate/SCFA	Propionate/SCFA	SCFA/BCFA[c]
Enterobacteriales	-0.25*	-0.32**		-0.28**	0.22*	
Fusobacteriales					0.42**	
Lactobacillales						-0.21*
Verrucomicrobiales						-0.22*
Family						
Bacteroidaceae			**0.53****		**0.54****	0.28**
Bifidobacteriaceae	0.32**				-0.31**	
Coriobacteriaceae			-0.21*		-0.25*	
Enterobacteriaceae	-0.25*	-0.32**		-0.28**	0.22*	
Eubacteriaceae				0.22*		
Incertae Sedis XI	0.21*					
Incertae Sedis XIV	0.4**	0.36**		0.21*		0.34**
Peptostreptococcaceae						-0.3****
Porphyromonadaceae			**0.46****		**0.51****	
Ruminococcaceae	0.29**	**0.48****	0.24*	0.39**		0.4**
Streptococcaceae						-0.2*
Veillonellaceae	0.3**	0.22*	**0.6****		**0.46****	
Genus						
Actinomyces	-0.26**					0.21*
Akkermansia						-0.22*
Alistipes					0.22*	
Anaerovorax						-0.2*
Asticcacaulis			0.23*		0.2*	
Atopobium						
Bacteroides			**0.52****		**0.54****	
Bifidobacterium	0.32**				-0.31**	
Blautia	0.4**	0.36**				

Table 2.4 (continued). Correlation between bacterial metabolites and bacterial populations.[a b]

	Total SCFA	Butyrate	Propionate	Butyrate/SCFA	Propionate/SCFA	SCFA/BCFA[c]
Citrobacter						
Clostridium				0.2*	0.2*	
Collinsella			-0.21*		-0.25*	
Coprococcus			**0.47****		**0.52****	
Eggerthella	**-0.45****					
Enterobacter						0.32**
Escherichia/Shigella	-0.25	-0.33**		-0.28**	0.23**	
Eubacterium		0.23*		0.22*		
Faecalibacterium	0.36**	**0.63****		**0.54****		0.41**
Gordonibacter	-0.31**					
Gp25						0.23*
Klebsiella						0.38**
Lactococcus	-0.3					
Megasphaera			0.23*			
Mitsuokella						
Mogibacterium					0.27**	
Odoribacter						
Olsenella					0.34**	
Oscillibacter			0.35**		0.28**	
Oxalobacter						
Paenibacillus						
Parabacteroides			**0.46****		**0.51****	
Paraprevotella						0.22*
Parascardovia						
Parasporobacterium						
Parasutterella						
Peptococcus					0.22*	0.23*

Table 2.4 (continued). Correlation between bacterial metabolites and bacterial populations.[a b]

	Total SCFA	Butyrate	Propionate	Butyrate/SCFA	Propionate/SCFA	SCFA/BCFA[c]
Phascolarctobacterium						
Phascolarctobacterium	0.29*	0.22*	**0.59****		0.4**	0.29*
Prevotella			0.22*			
Robinsoniella	-0.2*					
Roseburia	0.24*					
Samsonia			0.3*		0.2*	
Sarcina			0.24*			
Slackia	0.29**		0.23*			
Sporacetigenium						-0.29**
Staphylococcus						
Streptococcus						-0.2*
Subdoligranulum	-0.29**	-0.22*	-0.2*			
Succinivibrio					0.3**	
TM7_genera_incertae_sedis						
Tsukamurella						
Turicibacter						-0.2*
Others						
unclassified_"Bacteroidales"			0.7**		**0.77****	0.25*
unclassified_"Bacteroidetes"			0.37**			0.21*
unclassified_"Fusobacteriaceae"					0.42**	
unclassified_"Fusobacteriales"					0.31**	
unclassified_"Porphyromonadaceae"			0.21*		0.26*	
unclassified_"Prevotellaceae"			0.19*			
unclassified_Actinobacteria	0.35**				-0.33**	
unclassified_Actinobacteridae						
unclassified_Actinomycetaceae					0.31**	

Table 2.4 (continued). Correlation between bacterial metabolites and bacterial populations.[a b]

	Total SCFA	Butyrate	Propionate	Butyrate/SCFA	Propionate/SCFA	SCFA/BCFA[c]
unclassified_Betaproteobacteria			**0.59***		0.33**	
unclassified_Bifidobacteriaceae	0.33*					
unclassified_Enterobacteriaceae					0.29**	
unclassified_Incertae Sedis XIV		0.25*				
unclassified_Veillonellaceae			0.29**		**0.52****	

[a]Only significant correlations are presented; * significance at $p < 0.05$; **significance at $p < 0.01$; correlation coefficients greater than $|r| = 0.45$ are bold-printed; [b]Bacterial taxa where the sum of population in all 15 subjects <1% were excluded; [c]SCFA: Short chain fatty acid; BCFA: branched chain fatty acid.

Table 2.5.Correlation between carbohydrate utilization and bacteria population. [a b]

	Uronic acid in pectin	Guar gum	Inulin	AX	β-glucan	RS-2
Phylum						
Actinobacteria						0.66*
Proteobacteria		-0.52*			-0.64**	-0.66*
Firmicutes					**0.73****	
Class/subclass						
Actinobacteria						0.66*
Gammaproteobacteria		-0.53*			-0.64**	-0.62*
Erysipelotrichi					0.69**	
Actinobacteridae						0.56*
Order						
Erysipelotrichales					0.69**	
Enterobacteriales		-0.53*				-0.62*
Bifidobacteriales						0.56*
Family						
Eubacteriaceae	**-0.73****					
Bacteroidaceae						
Enterobacteriaceae		-0.53*			-0.64**	-0.62*
Bifidobacteriaceae						0.56*
Erysipelotrichaceae					0.69**	
Lactobacillaceae		-0.58*				
Genus						
Bifidobacterium						0.56*
Subdoligranulum			**-0.73****			
Escherichia/Shigella		-0.55*			-0.64**	-0.63*
Coprobacillus					0.54*	

Table 2.6.Correlation between carbohydrate utilization and bacteria population. [a] [b]

	Uronic acid in pectin	Guar gum	Inulin	AX	β-glucan	RS-2
Eubacterium	**-0.74****					
Others						
unclassified_"Bacilli"					0.58**	
unclassified_Actinobacteridae			0.56*			
unclassified_Bifidobacteriaceae						0.59*

[a] Only significant correlations are presented; *significance at $p < 0.05$; **significance at $p < 0.01$; correlation coefficients greater than $|r| = 0.45$ are bold-printed; [b]Bacterial taxa where the sum of population in all 15 subjects <1% were excluded; [c]SCFA: Short chain fatty acid; BCFA: branched chain fatty acid.

Table 2.7. Differences in the fecal microbiota obtained from normal weight (n=7) and obese (n=8) individuals [a,b]

	Normal weight	Obese	P-value
Phylum			
Firmicutes	81.8±4.16	74.99±4.82	0.31
Bacteroidetes	6.67±3.15	9.57±3.90	0.58
Proteobacteria	4.16±3.68	3.28±1.59	0.82
Actinobacteria	4.35±1.01	9.49±3.08	0.16
Verrucomicrobia	1.69±1.44	1.51±1.42	0.93
Synergistetes	0.12±0.12	ND [c]	0.3
Class			
Clostridia	78.11±4.21	65.81±3.97	0.053
Erysipelotrichi	1.98±0.38	6.68±2.31	0.084
Order/suborder			
Bifidobacteriales	1.26±0.37	6.27±2.35	0.071
Clostridiales	77.75±4.23	65.72±3.99	0.059
Erysipelotrichales	1.98±0.38	6.66±2.31	0.084
Class/subclass			
Actinobacteridae	1.28±0.37	6.31±2.35	0.0705
Family /subfamily			
Incertae Sedis XIV	22.02±3.83	7.75±1.43	**<0.0001**
Lachnospiraceae	27.05±1.57	18.18±3.30	**0.04**
Bifidobacteriaceae	1.26±0.37	6.27±2.35	0.071
Erysipelotrichaceae	1.98±0.38	6.66±2.31	0.084
Eubacteriaceae	0.43±0.31	9.02±4.13	0.076
Clostridiaceae	0.097±0.04	0.018±0.013	0.098
Genus			
Blautia (Ruminococcus)[d]	21.96±3.84	7.70±1.43	**<0.0001**
Bifidobacterium	1.22±0.36	6.02±2.28	0.073
Eubacterium	0.76±0.42	8.99±4.14	0.076
Others			
unclassified_Bifidobacteriaceae	0.02±0.02	0.24±0.08	**0.03**

[a]Values are presented as percent; mean ± SE (n=7 in normal weight group and n=8 in obese group); [b]T-test was used to compared the different bacterial taxa between obese and normal weight individuals; all differenced among bacteria at the phylum level are reported; for other classifications, only bacterial groups where p for difference was <0.1 are reported. Significant p-values (<0.05) are in bold; [c]not detected; [d]some or all members of *Ruminococcus* have been reassigned to *Blautia* on the basis of 16S rRNA gene sequence.

Table 2.8. Difference in abundance of bacterial taxa between obese and normal weight group after dietary fiber treatments.[a,b]

	Control		Pectin		Guar gum		Inulin	
	Normal	Obese	Normal	Obese	Normal	Obese	Normal	Obese
Phylum								
Actinobacteria	1.54±0.36	7.14±2.15	30.48±10.31	46.51±11.53	2.99±0.75	6.88±2.08	15.31±8.47	25.28±6.08
Class/Subclass								
Bacilli	3.91±0.87	5.28±2.42	18.35±10.95	1.49±0.69	11.15±8.85	1.27±0.36	**9.86±4.25**	**1.05±0.53**
Actinobacteria	1.54±0.36	7.14±2.14	30.49±10.31	46.51±11.53	2.99±0.75	6.88±2.08	15.31±8.47	25.28±6.08
Actinobacteridae	0.76±0.41	4.40±1.50	22.56±10.15	36.39±12.07	1.72±0.80	5.18±1.50	5.86±2.82	13.09±5
Order								
Bifidobacteriales	0.74±0.41	4.34±1.38	22.54±10.14	36.33±12.06	1.70±0.79	5.16±1.49	5.84±2.81	13.07±5
Family								
Bifidobacteriaceae	0.74±0.41	4.35±1.37	22.54±10.14	36.35±12.06	1.70±0.79	5.16±1.49	5.84±2.81	13.07±5
Genus								
Coprobacillus	0.59±0.2	1.15±1.03	**4.58±2.11**	**0.27±0.13**	0.16±0.12	0.04±0.02	10.19±7.34	0.9±0.47
Anaerotruncus	0.08±0.03	0.09±0.05	0.05±0.02	0.03±0.02	0.06±0.02	0.03±0.02	**0.03±0**	**ND**
Butyricicoccus	0.29±0.11	0.18±0.07	0.08±0.05	0.04±0.02	0.39±0.11	0.66±0.29	**0.19±0.08**	**0.03±0.01**
Catenibacterium	0.25±0.19	1.61±0.94	0.05±0.03	8.85±5.66	1.87±1.57	2.85±1.35	**0.69±0.55**	**25.19±9.96**
Bifidobacterium	0.72±0.40	4.23±1.34	22.10±9.98	35.52±11.83	1.66±0.76	5.07±1.47	5.69±2.74	12.82±4.91
Unclassified								
unclassified_"Clostridia"	0.03±0.01	0.03±0.02	**0.06±0.02**	**ND**	0.32±0.14	0.01±0.006	**0.02±0.008**	**0.001±0.001**
unclassified_"Bacilli"	0.03±0.01	0.09±0.06	0.05±0.05	0.005±0.005	0.02±0.009	0.03±0.01	**0.02±0.007**	**ND**
unclassified_Bifidobacteriaceae	0.02±0.009	0.12±0.04	0.44±0.18	0.83±0.24	0.05±0.03	0.09±0.03	0.15±0.08	ND
unclassified_Clostridiales	3.34±0.46	3.08±0.79	0.004±0.004	ND	2.08±0.44	2.84±0.56	0.003±0.003	ND

Table 2.7 (continued). Difference in abundance of bacterial taxa between obese and normal weight group after dietary fiber treatments.[a,b]

	AX		β-glucan		RS-2	
	Normal	Obese	Normal	Obese	Normal	Obese
Phylum						
Actinobacteria	**2.87±0.7**	**9.27±2.45**	1.4±0.37	6.80±2.62	6.11±2.94	23.92±8.46
Class/Subclass						
Bacilli	3.11±1.37	3.39±1.83	13.83±8.66	1.12±0.44	17.63±10.64	1.18±0.72
Actinobacteria	**2.87±0.70**	**9.28±2.46**	1.40±0.37	6.80±2.62	6.11±2.94	23.92±8.46
Actinobacteridae	**1.92±0.65**	**7.75±2.3**	**0.58±0.20**	**2.03±0.56**	1.92±1.22	18.13±8.06
Order						
Bifidobacteriales	**1.91±0.64**	**7.74±2.30**	**0.57±0.20**	**2.02±0.56**	1.92±1.22	18.08±8.04
Family						
Bifidobacteriaceae	**1.91±0.64**	**7.74±2.30**	**0.57±0.20**	**2.02±0.56**	1.92±1.22	18.08±8.04
Genus						
Coprobacillus	2.23±0.74	1.65±1.02	2.39±1.10	6.49±2.40	3.17±2.42	1.68±1.32
Anaerotruncus	0.06±0.02	0.05±0.03	20.48±7.71	5.95±3.21	0.26±0.01	0.07±0.05
Butyricicoccus	0.16±0.05	0.11±0.04	0.03±0.02	0.01±0.007	0.09±0.06	0.10±0.008
Catenibacterium	0.16±0.09	2.30±1.40	0.09±0.03	0.14±0.08	3.58±2.31	4.37±2.33
Bifidobacterium	**1.90±0.64**	**7.61±2.26**	**0.55±0.19**	**1.97±0.55**	1.86±1.18	17.68±7.87
Unclassified						
unclassified_"Clostridia"	0.08±0.05	0.18±0.008	0.004±0.002	0.009±0.005	0.19±0.01	ND
unclassified_"Bacilli"	0.006±0.004	0.24±0.01	0.003±0.002	0.0051±0.003	0.06±0.03*	0.0025±0.001
unclassified_Bifidobacteriaceae	0.02±0.009	0.13±0.04	0.02±0.01	0.039±0.016	0.05±0.03	0.40±0.18
unclassified_Clostridiales	3.25±0.84	2.50±0.55	1.88±0.21	4.10±0.90	1.55±0.60	1.89±0.64

[a] Values are reported as mean ± SE (n=7 in normal weight group and n=8 in obese group); [b] Within each dietary fiber treatment, t -test was used to compared the bacteria population. Only taxa that reach statistical significance were reported. Numbers in bold represent proportions that were significantly difference between obese and normal group. Significance at $p < 0.05$.

CHAPTER 3. IN VITRO FERMENTATON OF DIETARY FIBERS FROM SELECTED WHOLE GRAINS USING FECAL MICROBIOTA FROM OBESE AND NORMAL WEIGHT INDIVIDUALS

3.1. Abstract

Aberrations in the gut microbiota have been associated with obesity and other metabolic diseases. One way that gut bacteria may influence metabolism is through their metabolites [mainly short chain fatty acids (SCFA)], which can influence hormones involved in energy absorption, utilization, and storage. Epidemiological evidence suggests that obese individuals generally consume less whole grains than normal weight individuals; thus whole grains may improve the metabolic function of the aberrant microbiota in obese individuals and improve health. Five whole grains [wheat, rye, maize (corn), rice, and oats] were subjected to *in vitro* digestion and fermentation using fecal samples from eight obese and seven normal weight people by leaving fecal samples separate and by combining all fecal samples from each group to make normal weight and obese composite microbiota (NCM and OCM, respectively). When fecal samples were kept separate, large differences were observed in SCFA and gas production among individuals, even within group. When fecal samples from each group were combined, from 0-6 h, bacteria in NCM produced more SCFA than the OCM (10.95±1.82 vs. 0.83±0.13 μmol/h/100mg carbohydrate, respectively, $p < 0.0001$); whereas during 12-24 h, the OCM produced more SCFA than the NCM (12.15±3.19 vs. 7.74±1.41 μmol/h/100mg carbohydrate, respectively, $p<0.035$). Butyrate production from the NCM was significantly higher than from the OCM, while propionate production from OCM was significantly higher than NCM. These data confirm that there are large differences in fermentation profiles among individuals, even when supplied with the same dietary fiber; however, using composite microbiota it was apparent that the OCM was less metabolically active than the NCM initially, but that given sufficient whole grain substrates

the bacteria quickly bacome metabolically active. The OCM was also less butyrogenic than the NCM.

3.2. Introduction

Globally, 300 million people are obese and more than 100 billion are overweight [1]. Obesity is a serious public health concern, since it leads to conditions such as heart disease, stroke, type 2 diabetes and certain types of cancer [2]. Therefore, new insight and strategies on preventing obesity are becoming increasingly urgent.

Recently, the association between the gut microbiota and obesity has been a subject of intense investigation. The human gut is a complex microbial ecosystem containing about 100 trillion microorganisms that may play pivotal roles in energy absorption [3], utilization [4], and storage [5-7]. There is increasing evidence that the relative proportions of members of the gut microbiota are associated with obesity [8, 9]. Changes in the gut microbiota have resulted from dietary intervention and weight loss [10, 11].

The gut microbiota survive in large part on dietary fibers that escape digestion in the upper gastrointestinal tract. Fermentation of dietary fibers by gut bacteria result in, among other products, short chain fatty acids (SCFA). Normal colonic epithelia cells derive 70-90% of their energy supply from SCFA, especially butyrate [12]. Fifty to seventy percent of acetate is taken up by the liver and becomes a substrate for cholesterol synthesis; propionate is also largely taken up by the liver and is incorporated into gluconeogenesis, liponeogenesis and protein synthesis pathways [13, 14].

SCFA contribute to various metabolic processes, accounting for about 10% of total energy harvest from food in humans [15]. However, SCFA induce a satiety-enhancing effect which has been suggested to offset the increased energy they provide [16, 17]. Furthermore, SCFA may play key roles in regulation of hormones involved in energy metabolism. For instance,

two G-protein couple receptors, GPR43 and GPR41, can be activated by SCFA [18-20]. Activation of GPR41 leads to up-regulation of leptin and peptide YY (PYY), hormones that reduce appetite [21, 22]. SCFA may also activate proliferator-activated receptor-α (PPAR-α), a transcription factor that is involved in fatty acid, lipoprotein, bile acid and amino acid metabolism. Thus one way the gut microbiota may influence energy metabolism is through the production of SCFA from carbohydrate fermentation [23].

Epidemiological studies have found there is an inverse association between consumption of whole grain and obesity [24]. Whole grains are a good source of dietary fiber and many unique bioactive phytochemicals that may influence metabolism of microbiota in favor of weight reduction. Thus, the purpose of this study was to compare SCFA production from fecal microbiota obtained from obese and normal weight individuals upon whole grain fermentation in an effort to explain how whole grains may reduce obesity.

3.3. Materials and Methods

3.3.1. Whole grains

Whole grain brown rice (*Oryza sativa* L.) was obtained from Riceland Foods, Inc. (Stuttgart, AR USA). Maize (*Zea mays* L., referred to herein as “corn”) and hard red winter wheat (*Triticum aestivum* L.) were gifts from Stephen Mason (University of Nebraska-Lincoln) and P. Stephen Baenziger (University of Nebraska-Lincoln), respectively. Oat groats (*Aveba sativa* L.) and dark rye flour (*Secale cereale* L.) with about 10% added bran were provided by ConAgra Mills (Omaha, NE USA) and Grain Millers Inc. (Minneapolis, MN USA) respectively. Oats, wheat, and brown rice were milled with a cyclone sample mill (UDY, Boulder, CA USA) equipped with a 1 mm screen. Corn was milled on a micro hammer mill (Glen Mills, Clifton, NJ USA) equipped with a 2 mm screen. Whole grains were analyzed for moisture according to AACC International 44-15A method [25], starch (K-TSTA, Megazyme, Bray, Ireland) and total dietary fiber (see below).

3.3.2. Fecal samples

Fifteen selected fecal samples were obtained from Rush University Medical Center as described in section 2.3.1.

3.3.3. *In vitro* digestion

The digestion process consisted of a simulated gastric digestion followed by a small intestinal phase following the method as described in section 2.4.1.

3.3.4. *In vitro* fermentation

In vitro fermentation followed two methods (Figure 3.1) based on whether the fermentation was performed without combining fecal samples (method 1) or whether the fecal samples from normal weight individuals or obese individuals were pooled to produce composite microbiota (NCM = normal composite microbiota; OCM = obese composite microbiota; method 2). Small scale *in vitro* fermentation was completed as described in section 2.4.2 using 10 mg total carbohydrate per tube (method 1) or 40 mg of carbohydrate per tube (method 2) with buffer and media volumes increased proportionally. For method 1, samples were taken at 12 h by plunging in an ice bath and then freezing (-80 °C) until analysis. For method 2, samples were taken at 6, 12, and 24 h of fermentation in a similar manner. Prior to taking samples, the volume of gas produced by the bacteria was measured by inserting a lubricated glass syringe with needle through a septum in the cap of the tube.

3.3.5. Total dietary fiber and total carbohydrate

Total dietary fiber and total carbohydrate were analyzed in whole grain samples before and after *in vitro* digestion, respectively. The dietary fiber content was determined as sum of neutral sugar residues, uronic acid residues and Klason lignin; total carbohydrate was the sum of neutral sugar and uronic acid residues. Neutral sugars and uronic acids were quantified according

to the method as described in section 2.3.5. Klason lignin was determined as described by Theander and Westerlund [26].

3.3.6. Short chain fatty acids

Short chain fatty acids (SCFA) were quantified following the same protocol as described in section 2.3.6.

3.4. Statistical analysis

For method 1, fecal donor served as replication within weight group (obese vs. normal weight); 6 tubes were fermented per donor (1 for each whole grain plus the blank containing no carbohydrate). For method 2, the fermentation tube itself served as a replicate, which was performed in duplicate. Thus, 12 tubes were fermented per composite microbiota (2 for each whole grain plus 2 for the blank). The effects of different whole grains on SCFA and gas production were determined using analysis of variance (ANOVA) followed by Fisher's protected least significant difference using SAS software (version 9.2, SAS Institute, Cary, NC USA) with α=0.05 to indicate a significant difference. Data were expressed as mean±standard error.

3.5. Results and discussion

3.5.1. Total carbohydrate content

The starch content and dietary fiber content of five whole grain starting materials are shown in Table 3.2. Before subjecting the whole grain to *in vitro* fermentation, *in vitro* digestion procedure was performed to mimic the digestion process occurring in the gastrointestinal tract. After *in vitro* digestion, starch content was removed to under 4.5% on all five substrates (Table 3.2). Considering that the resistant starch content of whole grain is about 3.3% [27], we concluded that the *in vitro* digestion procedure was successful at removing the digestible starch [26, 27].

3.5.2. Gas production (method 1)

No significant differences in gas production were observed between obese and normal weight group (Table 3.2). The least gas production was detected from both groups on corn substrate.

3.5.3. SCFA production from individual fecal microbiota (method 1)

In vitro fermentation of whole grains resulted in the production of gas and SCFA (Table 3.2). Minor fermentation occurred in the blank (without substrate) due to fermentation of residual substrates in the inoculum. Among the different whole grain substrates, butyrate production was significantly higher on rye substrate and propionate production was significantly higher on rice substrate compared with other cereal substrates. Unfortunately, no significant differences in metabolite production were observed between normal weight individuals compared with obese individuals for any of the whole grain samples. The reason for this is evident in Figure 3.2, which shows large differences in SCFA production among individuals, even within weight group. This supports previous research showing that individuals harbor gut microbial communities whose species composition and relative proportions of dominate microbial groups are tremendously varied due to host genetic and environmental factors [30].

There appeared to be an upward trend in SCFA production with increasing BMI in obese group (Figure 3.2); however, correlation coefficients were not significant (all obese data combined: $p=0.10$, for individual whole grain: corn, $p=0.23$; oats, $p=0.37$; rice, $p=0.78$; rye, $p=0.42$; and wheat, $p=0.61$). Were the trend significant, this would have supported Schwiertz et al. [31] who reported higher SCFA concentrations in obese fecal samples compared with normal weight fecal samples. Ley et al. [32] also observed higher SCFA in caeca of obese mice compared with lean mice.

In the dietary fiber literature, it is common to pool fecal samples to reduce individual variation in fecal microbiota to more clearly gauge differences in fermentation profiles among different dietary fiber substrates [33-34]. We hypothesized that combining fecal samples from

each group may help decipher differences between obese and normal weight microbiota metabolic products by reducing individual variation. When combining the fecal samples, although biases arise because of the competition between gut microbiota from different individual fecal sources, to some degree it can represent the relative whole picture and characteristics of normal or obese type microbiota [35]. This was the reason for performing method 2 (discussed below).

3.5.4. Gas production (method 2)

For method 2, the gas produced from NCM was significantly higher at all-time points on all substrates, except for rice at 6 h (Figure 3.3). Higher gas production in NCM was associated with higher metabolic activity, which is in accordance with SCFA data (discussed below) and could be regarded as an indication that the bacteria were more metabolically active in the normal weight group. On oats and rye substrates more gas was produced from NCM compared with other substrates, which could be due to higher soluble dietary fiber content in oats and rye compared with other whole grain [36, 37]. Higher β-glucan content in oats has been associated with bloating in some cases [38]. Generally, wheat produced less gas in NCM and OCM compared with other whole grains which suggest less potential of causing bloating.

3.5.5. SCFA production from composite fecal microbiota (method 2)

When comparing the average SCFA production from NCM and OCM during different fermentation periods, significant differences were observed. At the beginning of fermentation (0-6 h), the rate of SCFA production in the NCM was significantly higher (about 15 times higher) than the OCM (Figure 3.4), whereas during the final half of fermentation (12-24 h), the rate of SCFA production was significantly higher in the OCM compared with the NCM. Thus, the OCM showed a longer lag period than the NCM.

These results are at first in opposition to Schwiertz et al. [4] and Ley et al. [31] who reported greater SCFA production in the feces (human) and cecum (mice) of obese compared

with normal weight individuals, respectively. However, it is possible that these results are in accordance with at least Schwiertz et al. [4] when considering that the obese microbiota may display protracted fermentation compared with normal weight individuals; thus delaying fermentation and resulting in higher fecal SCFA. Reports that obese individuals consistently consume less dietary fiber-rich foods than normal-weight individuals in epidemiological studies [39] may explain why the OCM were in more of a dormant state during the initial several hours of fermentation. Notably, however, the OCM was able to metabolize the whole grain substrates after sufficient exposure such that total SCFA production was not significantly different from the NCM after 24 h (250.14±49.46 μmol /100mg carbohydrate vs. 259.40±39.47 μmol /100mg carbohydrate, respectively, p for difference =0.62).

By combining fecal samples from each weight group, significant differences in individual SCFA production were evident between NCM and OCM (Table 3.3). After 6 h of fermentation, NCM produced more acetate than OCM regardless of whole grain substrate. Surprisingly, no propionate or butyrate production from NCM was detected during this stage of fermentation, and amounts of butyrate and propionate detected from OCM were not significantly different from zero ($p>0.05$). This may be a result of starting the *in vitro* cultures with frozen fecal samples; some bacteria needed time before becoming metabolically active after being frozen [40]. It is also possible that other factors such as pH of the medium may have affected the production of SCFAs. The pH of the initial medium was about 7.4; many saccharolytic bacteria thrive better under more acidic conditions such as between 5 and 6 [41]. Also, acetate producing bacteria instead of butyrate or propionate producing bacteria may have initially competed more successfully for the utilization of whole grain substrates [42]. Kaur et al. [43] also reported some negative propionate values compared with the controls at 4 and 8 h under *in vitro* fermentation conditions.

After 12 h of fermentation, the NCM continued to produce more acetate than OCM on all five whole grain substrates (Table 3.3). OCM produced significantly more propionate compared

with NCM on all substrates except rye, whereas butyrate production was not significantly different between NCM and OCM except on wheat substrate.

Also at 12h of fermentation, significantly less propionate production from OCM on all whole grains except corn was detected compared with NCM, while after extended fermentation of whole grain substrates more propionate was observed from OCM except on corn substrate (Table 3.3). This is in support of Schwiertz et al. [31], who showed an enrichment in propionate in fecal samples from obese individuals compared with normal weight.

At the end of the fermentation (24 h), butyrate from NCM was higher than from OCM except on corn substrate. This may have been a result of lower pH in the NCM slurries, brought about by the decreased pH as a result of rapid acetate production by the NCM at the beginning of fermentation, which supported the formation of butyrate by allowing butyrate-producing bacteria to compete against those that are less prone to butyrate production [45].

Butyrate production was highest when NCM was on oats substrate compared with other whole grain (Table 3.4). This is in accordance with the finding of Queenan et al [38] and Connolly et al [46] showing that fermentation of oats leads to significant increase in butyrate production.

Butyrate exhibits many benefits on colon health. It has been reported that 70%-90% of butyrate is metabolized by colonocytes [47]. As the preferred energy source of colonocytes, butyrate has also been implicated in the control of the machinery regulating apoptosis and cellular proliferation and differentiation, therefore decreases the risk of colon cancer. At the intestinal level, butyrate plays a regulatory role in the transepithelial fluid transport, ameliorates mucosal inflammation and oxidative status, reinforces the epithelial defense barrier, and modulates visceral sensitivity and intestinal motility [46]. Butyrate supplementation in obese mice significantly reduced obesity and insulin resistance. The underlying mechanism of butyrate action may due to stimulation of energy expenditure and induction of mitochondria function [49]. Other

research also reported that butyrate may promote satiety through inhibition of nuclear factor-κB activation, although human data are still limited [50].

3.5.6. BCFA production from composite fecal microbiota (method 2)

With regard to BCFA, more production from NCM was detected at the end of the fermentation though on some substrates the difference was not significant (Table 3.4). BCFAs result from amino acid fermentation. No studies have shown the detrimental effects caused by BCFAs themselves, although production of undesirable toxic metabolites and BCFAs often couple with each other [29, 51]. The higher BCFAs produced from the NCM may result from the induction of higher bacterial metabolism, which was also suggested by the SCFA production data.

3.5.7 Comparison between method 1 and method 2

When comparing the results from method 1 and method 2, some contradictory results arise. Method 1 suggested ($p=0.10$) an increasing trend in SCFA production after 12 h of fermentation as BMI increased in the obese group. This is in accordance with several reports showing greater metabolic activity in obese microbiota compared with normal weight [31,52]. Conversely, method 2 showed that the OCM were more sluggish than normal weight microbiota, producing far fewer SCFA during the first half of fermentation compared with NCM. When considering that obese individuals generally consume less dietary fiber than normal weight individuals, it may be that the obese microbiota are less active due to less substrate availability *in vivo*. Thus, more research on the metabolic and functional differences in the microbiota between obese and normal weight humans is necessary.

Among individual SCFAs produced, no significant differences were found in the butyrate production with method 1, while with method 2 the NCM produced more butyrate than the OCM. The lack of a statistical difference in the former case may simply be due to an insufficient n.

Notably, we only analyzed the SCFA production at the end of 12 h in method 1, while SCFA production at 6, 12, and 24 h were quantified in method 2. At 12 h of fermentation in both

methods, no significant differences were observed in total SCFA production between the normal weight and obese groups (176.56±16.53 μmol/100mg carbohydrate vs. 194.99±16.98 μmol/100mg carbohydrate for method 1, respectively, p for difference=0.25). The differences discovered in method 2 were mostly a result of shorter (6 h) or longer (24 h) fermentation times (Figure 3.4 and Table 3.3). It may be that more consistent results would have been observed between methods 1 and 2 had fermentation samples been taken at multiple time points in method 1.

3.6. Conclusion

Whole grains have been associated with reduced obesity risk, though the influence on the gut microbiota remains largely unknown. Large variations exist in fermentation properties among microbiota from different individuals, even when given the same substrate. Thus, no differences in SCFA production with increasing BMI were observed. When fecal samples were combined, however, to produce composite microbiota from each weight group, those from normal weight individuals were more metabolically active at the onset of fermentation compared to those from obese individuals, while the converse was true for the latter stages of fermentation. Also, more butyrate production was found from NCM at the end of fermentation. Although large variation exists in the SCFA profile among different individuals, our study showed that different whole grains influence the NCM and OCM in different fashions and may therefore elicit different physiological effects on our health.

3.7. References

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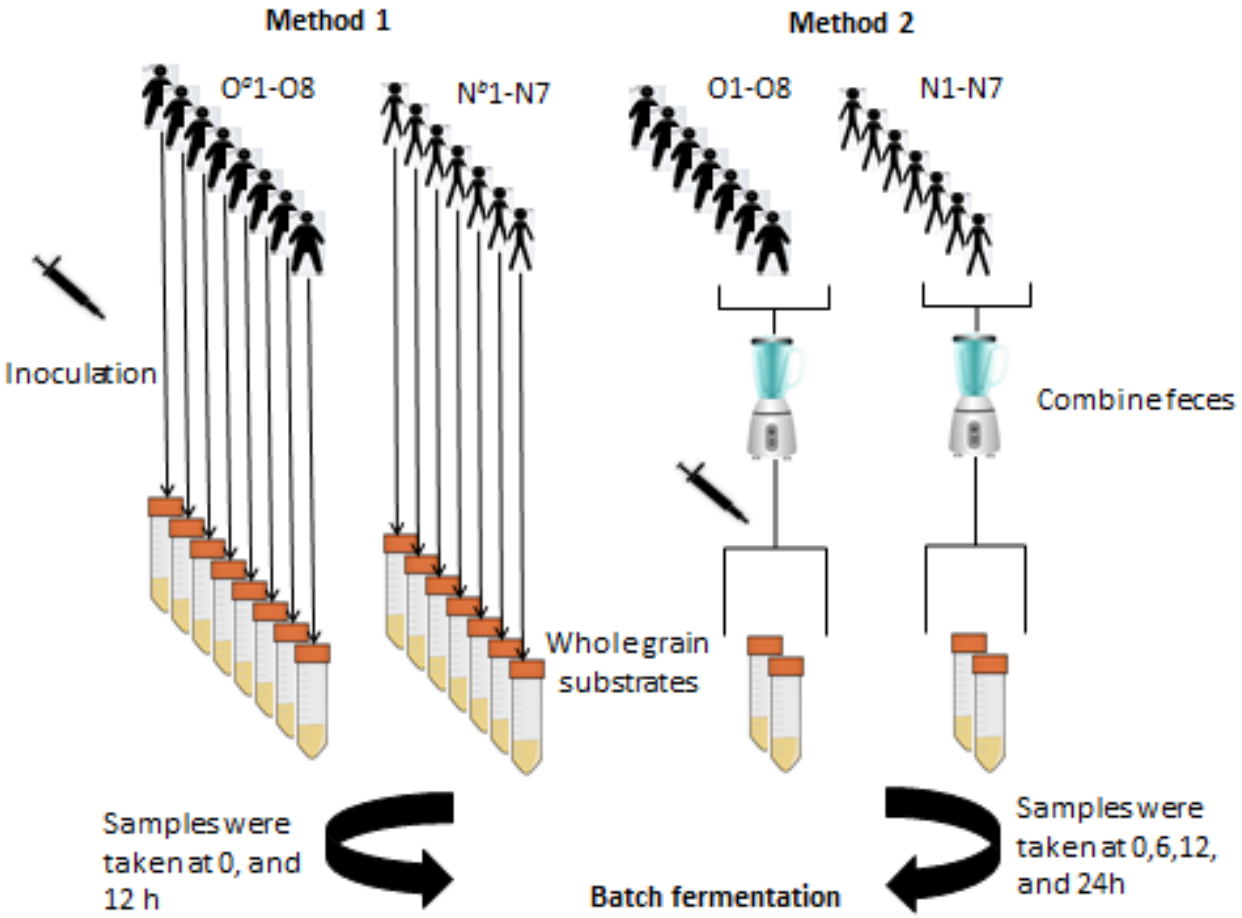

Figure 3.1. Graphical depiction of fermentations using method 1 and method 2; [a] fecal samples from obese individuals (n=8); [b] fecal samples from normal weight individuals (n=7).

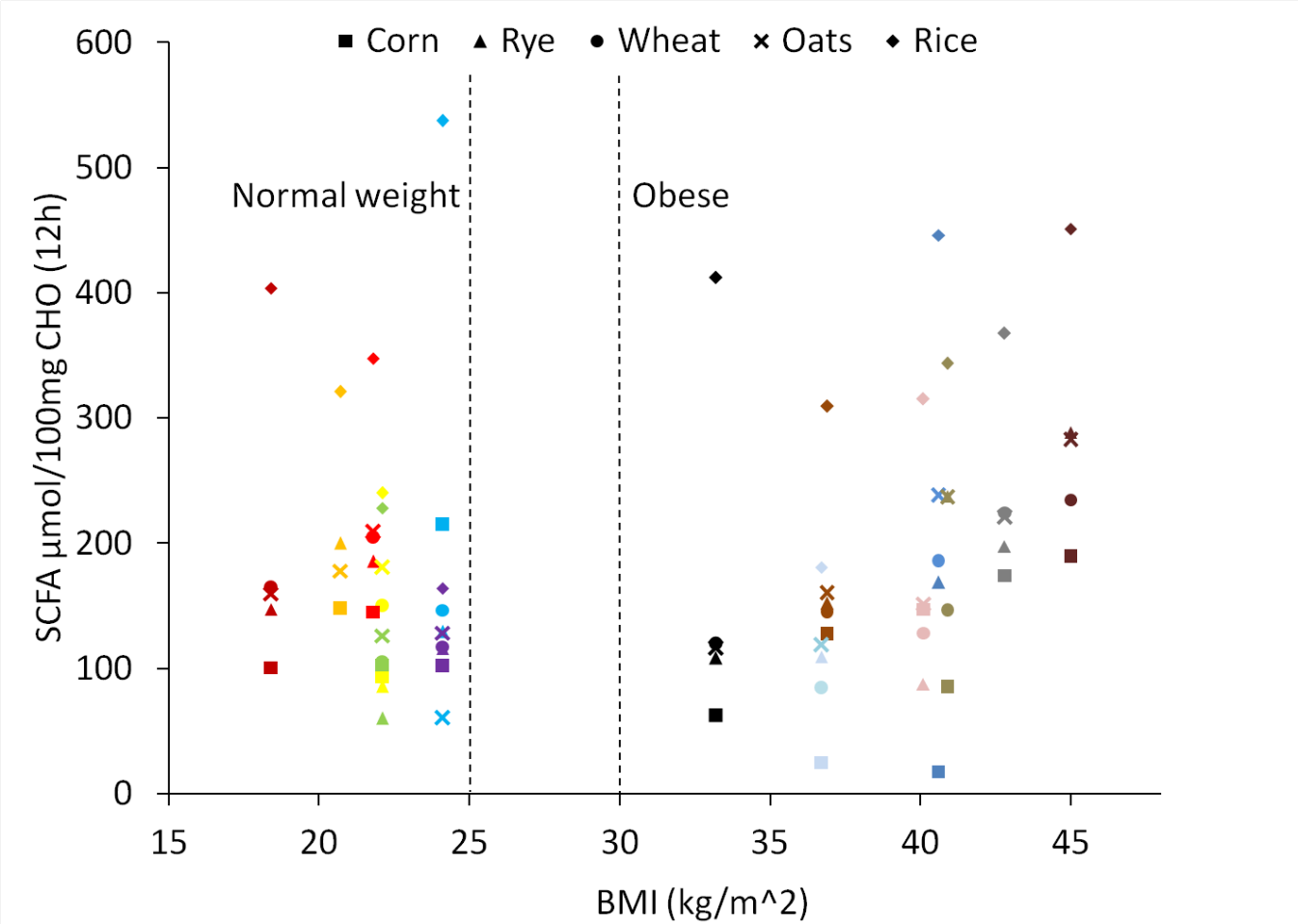

Figure 3.2. Short chain fatty acid (SCFA) production from each fecal sample on each dietary fiber substrate arranged by body mass index (BMI) of the person the fecal sample was collected from control containing no whole grain substrate has been subtracted.

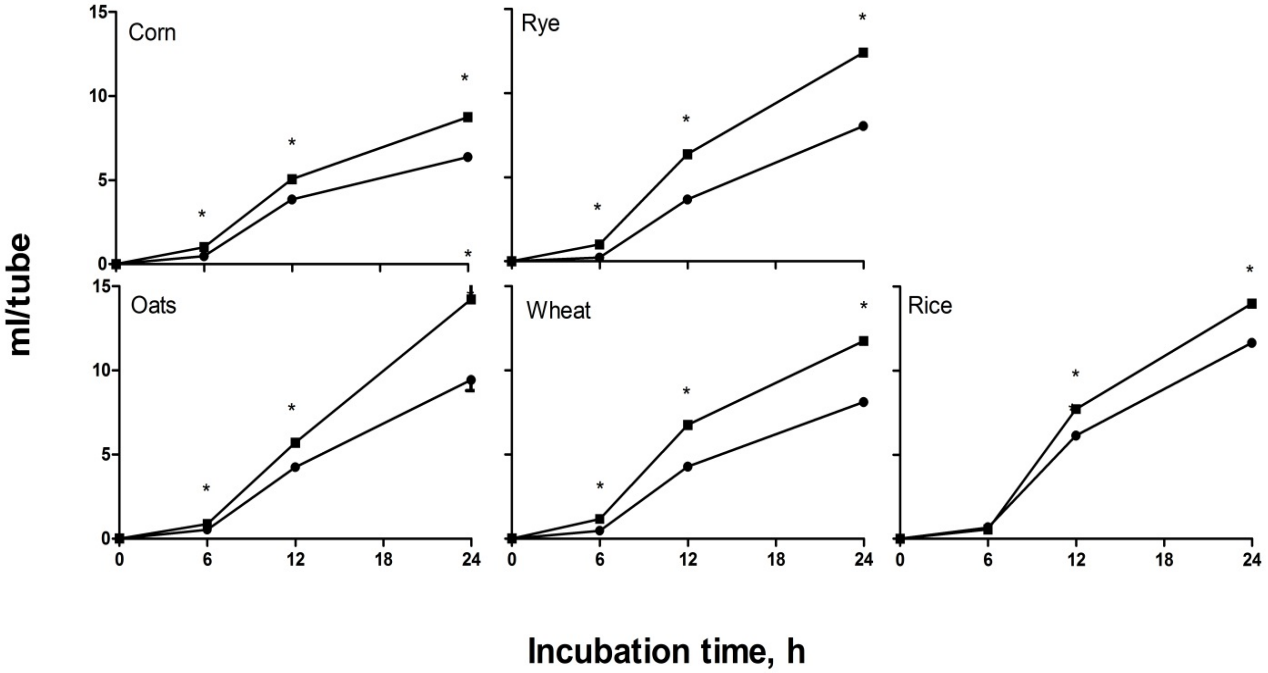

Figure 3.3.Gas production for corn, rye,oat, wheat, and rice; OCM (circles) and NCM (squares); values are presented as mean ± SE at each time point; * $p<0.05$ for difference from NCM at that time point.

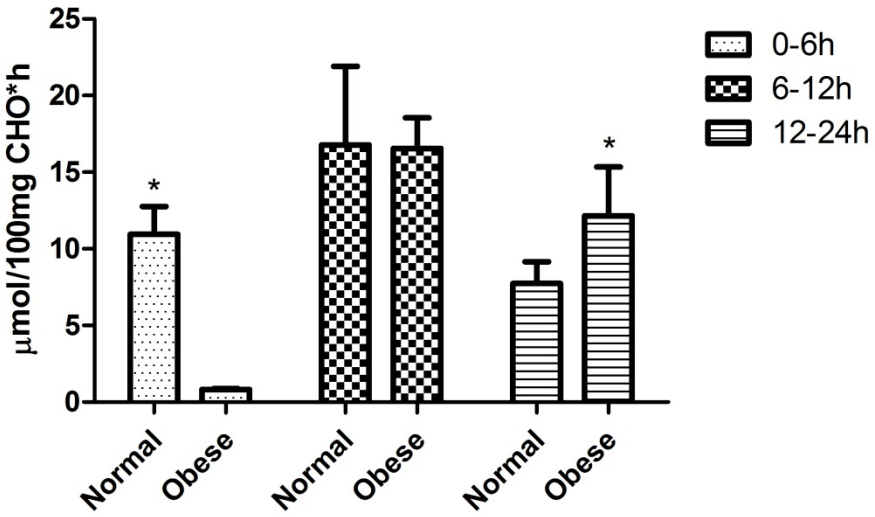

Figure 3.4. Rate of short chain fatty acid (SCFA) production during different stages of fermentation; values are presented as mean production from each weight group with data from all whole grain substrates combined; error bars show standard error; * indicates a significant difference at that time interval ($p<0.05$).

Table 3.1.Polysaccharide composition (%, db)[a] of whole grain samples before and after *in vitro* digestion.[b]

Whole grain sample	Starch%(db)	Dietary Fiber				Starch after *in vitro* digestion	Carbohydrate content after *in vitro* digestion(Neutral sugar+uronic acid)		
		Neutral Sugars	Uronic Acid	Lignin	Total		Neutral Sugars	Uronic acid	Total
Wheat	59.53± 0.067	9.22±0.51	1.29±0.07	2.04±0.02	11.58±0.54	4.49±0	38.31±0.38	1.38±0.03	39.68±0.35
Rice	75.41± 0.21	2.35±0.15	0.58±0.03	1.51±0.14	4.01±0.26	2.26±0.01	14.01±0.92	1.08±0.03	15.09±0.89
Oats	59.46± 0.55	7.24±0.07	1.14±0.06	2.38±0	9.90±0.11	2.57±0.05	26.60±0.10	1.89±0.05	28.49±0.05
Corn	63.27± 0.11	6.60±0.58	0.43±0.03	1.49±0.16	8.51±0.65	3.44±0.03	31.02±0.54	2.06±0	33.08±0.53
Rye[c]	33.58± 0.45	23.67±1.09	0.51±0.06	4.74±0.14	28.92±1.09	2.85±0.02	46.36±0.11	1.18±0.02	47.54±1.09

[a] db: dry basis
[b] Each value represents mean ±standard error of 2 replicates
[c] The rye used was not whole grain; it contained added bran

Table 3.2. Individual SCFA and BCFA[a] production (method 1)[b]

Whole grain	Group	Metabolite					
		Gas	Acetate	Propionate	Butyrate	Iso-butyrate	Iso-valerate
Control	Normal	1.13(0.13)^{d}	98.70(9.35)^{de}	9.57(2.38)^{d}	7.04(3.05)^{ab}	1.93(1.28)^{ns}	6.48(2.87)^{ab}
	Obese	0.86(0.13)^{d}	80.61(11.64)^{e}	7.92(1.94)^{d}	5.39(2.20)^{b}	1.24(1.17)^{ns}	2.67(7.38)^{b}
Corn	Normal	3.97(0.34)^{c}	215.95(21.41)^{bc}	27.71(9.55)^{abcd}	1.21(0.24)^{b}	2.39(1.67)^{ns}	7.25(3.30)^{ab}
	Obese	4.03(0.49)^{c}	167.05(19.71)^{cd}	28.24(10.13)^{abcd}	2.15(0.61)^{b}	0.56(0.24)^{ns}	6.47(10.24)^{ab}
Rye	Normal	4.73(0.37)^{bc}	218.91(20.40)^{bc}	11.81(3.31)^{cd}	16.60(5.23)^{a}	2.25(1.47)^{ns}	8.22(3.85)^{ab}
	Obese	4.82(0.34)^{bc}	230.15(25.7)^{bc}	16.53(4.58)^{cd}	15.92(6.56)^{a}	1.52(0.77)^{ns}	9.29(9.34)^{ab}
Oats	Normal	4.55(0.29)^{bc}	236.38(19.75)^{bc}	26.41(8.90)^{bcd}	1.37(0.36)^{b}	1.93(1.57)^{ns}	8.44(3.27)^{ab}
	Obese	4.81(0.55)^{bc}	241.65(31.77)^{b}	33.10(11.30)^{abc}	9.96(5.11)^{ab}	1.59(1.03)^{ns}	11.63(10.61)^{ab}
Wheat	Normal	4.68(0.42)^{bc}	245.38(18.20)^{b}	16.31(3.38)^{cd}	5.72(2.9)^{b}	2.07(1.35)^{ns}	7.20(2.73)^{ab}
	Obese	4.84(0.48)^{bc}	222.58(24)^{bc}	23.39(6.77)^{bcd}	6.63(2.14)^{b}	0.95(0.80)^{ns}	9.77(11.88)^{ab}
Rice	Normal	5.89(0.58)^{ab}	391.89(49.5)^{a}	41.85(11.09)^{ab}	1.87(0.34)^{b}	2.21(1.86)^{ns}	8.75(3.77)^{ab}
	Obese	6.42(0.89)^{a}	391.40(43.78)^{a}	49.58(10.47)^{a}	6.21(1.94)^{b}	3.88(2.00)^{ns}	15.62(20.55)^{a}

[a] SCFA: short chain fatty acid; BCFA: branched chain fatty acid.

[b] Values are reported as mean (standard error) of 2 replicates from each individual in the normal weight (n=7) and obese (n=8) groups; units for gas are ml/tube (100 mg initial carbohydrate); for SCFA and BCFA, units are μmol/tube (100 mg initial carbohydrate); values carrying different superscript letters within column are significantly different ($p<0.05$).

Table 3.3.Composite SCFA [a] production (method 2) [b]

Whole grain	Group	Metabolite								
		Acetate			Propionate			Butyrate		
		6h	12h	24h	6h	12h	24h	6h	12h	24h
Control	Normal	43.71(0.22)^{c}	104.18(2.07)^{g}	230.1(3.16)^{d}	ND$^{d\,C}$	6.05(2.22)^{def}	36.26(0.15)^{c}	ND^{c}	ND^{d}	20.93(0.29)^{bcd}
	Obese	8.12(0.05)^{d}	34.34(0.12)^{h}	73.83(20.37)^{e}	1.62(0.01)^{c}	6.19(0.1)^{def}	10.64(0.01)^{g}	1.2(0.39)^{bc}	2.56(0.18)^{bc}	2.14(0.28)^{d}
Corn	Normal	11.3.8(1.54)^{a}	187.55(6.37)^{d}	380.94(2.77)^{c}	ND^{d}	21.28(0.46)^{b}	47.88(1.85)^{b}	3.37(3.37)^{b}	1.50(0.14)^{bcd}	12.19(0.13)^{cd}
	Obese	11.59(1.18)^{d}	122.22(3.56)^{ef}	187.65(6.96)^{d}	2.44(0.29)^{a}	7.98(0.51)^{cde}	45.60(0.6)^{b}	1.54(0.14)^{bc}	1.38(0.05)^{bcd}	4.63(0.73)^{cd}
Rye	Normal	98.04(1.72)^{b}	210.41(0.58)^{c}	464.63(0.11)^{bc}	ND^{d}	7.23(0.51)^{cde}	19.07(0.33)^{f}	ND^{c}	1.37(0.04)^{bcd}	22.66(0.96)^{bc}
	Obese	12.71(0.71)^{d}	108.63(0.06)^{fg}	246.30(1.84)^{d}	1.88(0.15)^{bc}	8.75(0.17)^{cd}	29.60(0.07)^{d}	1.43(0.04)^{bc}	1.31(0.23)^{bcd}	1.58(0.10)^{d}
Oats	Normal	110.60(2.35)^{a}	219.09(9.66)^{bc}	461.95(38.41)^{bc}	ND^{d}	20.3(1.33)^{b}	28.57(2.39)^{d}	9.1(0.57)^{bc}	2.93(0.21)^{ab}	72.73(18.11)^{a}
	Obese	13.43(0.11)^{d}	132.05(3.21)^{e}	258.15(53.13)^{d}	2.03(0.01)^{b}	7.45(0.05)^{cde}	44.71(0.24)^{b}	1.22(0.25)^{bc}	1.17(0.14)^{cd}	5.86(1.08)^{cd}
Wheat	Normal	91.67(4.16)^{b}	229.95(4.87)^{bc}	402.61(24.61)^{c}	ND^{d}	10.83(0.50)^{c}	24.06(2.72)^{e}	ND^{c}	4.32(1.72)^{a}	35.39(3.07)^{b}
	Obese	10.8(0.25)^{d}	121.41(0.65)^{ef}	197.38(7.95)^{d}	1.87(0.04)^{bc}	4.26(0.05)^{fe}	36.89(0.66)^{c}	1.55(0.1)^{bc}	1.56(0.04)^{bcd}	2.75(1.70)^{d}
Rice	Normal	117.92(13.02)^{a}	432.39(4.07)^{a}	666.06(58.47)^{a}	ND^{d}	31.41(3.47)^{a}	39.33(0.56)^{c}	2.4(0.28)^{bc}	2.60(0.01)^{bc}	55.387(9.533)^{a}
	Obese	13.50(0.62)^{d}	214.42(11.19)^{bc}	518.39(28.05)^{b}	1.74(0.07)^{bc}	2.70(0.13)^{f}	64.26(0.25)^{a}	1.79(0.15)^{bc}	1.62(0.47)^{bcd}	6.62(2.08)^{cd}

[a] SCFA: short chain fatty acid;

[b] Values are reported as mean (standard error) of 2 replicates from each individual in the normal weight (n=7) and obese (n=8) groups; units for gas are ml/tube (100 mg initial carbohydrate); for SCFA and BCFA, units are μmol/tube (100 mg initial carbohydrate); values carrying different superscript letters within column are significantly different ($p<0.05$).

[c] none detected.

Table 3.4. BCFA [a] production (method 2) [b]

Whole grain	Group	Metabolite					
		Iso-butyrate			Iso-valerate		
		6h	12h	24h	6h	12h	24h
Control	Normal	2.7(0.13)^{ab}	2.90(0.22)^{a}	9.70(0.5)^{ab}	0.78(0.78)^{cd}	5.92(0.92)^{bc}	14.95(0.71)^{cd}
	Obese	0.14(0.14)^{ab}	ND^{b}	4.61(4.61)^{bc}	1.63(0.07)^{bc}	ND^{f}	5.70(4.12)^{defg}
Corn	Normal	2.84(2.84)^{a}	ND^{b}	10.78(0.58)^{ab}	ND^{d}	ND^{f}	27.71(1.01)^{ab}
	Obese	1.14(0.17)^{ab}	ND^{b}	0.67(0.19)^{c}	1.79(0.47)^{b}	0.81(0.23)^{ef}	2.81(0.16)^{fg}
Rye	Normal	0.81(0.81)^{ab}	2.75(0.01)^{a}	6.21(0.3)^{bc}	ND^{d}	8.32(0.28)^{b}	13.37(0.42)^{cde}
	Obese	1.02(0.01)^{ab}	0.65(0.65)^{b}	0.81(0.19)^{c}	1.03(0.08)^{bc}	1.68(0.12)^{def}	1.44(0.09)^{g}
Oats	Normal	ND^{b}	ND^{b}	6.19(0.58)^{c}	ND^{d}	3.11(0.89)^{cde}	18.28(1.26)^{bc}
	Obese	0.84(0.02)^{ab}	ND^{b}	0.86(0.06)^{c}	1.97(0.22)^{b}	1.15(0.08)^{def}	2.48(0.20)^{g}
Wheat	Normal	ND^{b}	1.04(0)^{b}	8.21(1.12)^{ab}	ND^{d}	3.62(0.94)^{cd}	12.20(4.81)^{cdef}
	Obese	0.63(0)^{b}	ND^{b}	0.78(0.09)^{c}	3.93(0.47)^{a}	2.09(0.53)^{def}	4.43(1.57)^{efg}
Rice	Normal	ND^{b}	4.04(1.46)^{a}	12.73(3.98)^{a}	ND^{d}	11.78(2.53)^{a}	33.28(7.42)^{a}
	Obese	0.46(0.06)^{ab}	ND^{b}	6.62(2.08)^{abc}	3.03(0.19)^{a}	ND^{f}	16.18(1.39)^{c}

[a] BCFA: branched chain fatty acid.
[b] Values are reported as mean (standard error) of 2 replicates from each individual in the normal weight (n=7) and obese (n=8) groups; units for gas are ml/tube (100 mg initial carbohydrate); for SCFA and BCFA, units are μmol/tube (100 mg initial carbohydrate); values carrying different superscript letters within column are significantly different ($p<0.05$).
[c] none detected

GENERAL CONCLUSIONS

The present thesis has reported the impact of dietary fiber and whole grain on the fecal microbiota from obese and normal weight individuals. The first hypothesis was that whole grain and dietary fibers may be an excellent way to treat the obesity by altering and modulating of the gut microbiota. The second hypothesis was that different whole grains and dietary fibers will exhibit varying efficacy with respect to correcting the abnormal gut microbiota in obesity.

For the first hypothesis, we only evaluated the impact of dietary fibers (and not whole grains) on gut microbiota due to limitations on the number of samples we could run. Our results showed that RS-2 caused the most dynamic change of the whole microbiota community and dietary fiber can influence the growth of certain bacterial taxa. For instance, *Bifidobacterium,* which has been shown to be more abundant in normal weight individuals [1] increased almost 10-fold on pectin. We also found that specific gut bacterial taxa were correlated with high SCFA production and/or utilization of certain dietary fibers. These taxa may be targeted to increase SCFA production or increase DF utilization. Also, there were significant differences in the gut microbiota between obese and normal weight individuals; each of these microbiota respond slightly different to the same dietary fiber.

For the second hypotheses, it was demonstrated that different whole grains and dietary fibers exhibit quite different effects on the both the composition and the metabolic profile of the gut microbiota. Also, we found that there were large differences in fermentation profiles among individuals, even when supplied with the same dietary fiber or whole grain. The obese type microbiota was less efficient at butyrate production, and the obese type microbiota was less metabolically active than the normal weight type microbiota initially, but that given sufficient whole grain substrates the bacteria quickly became metabolically active. Epidemiological evidences suggest that obese individuals generally consume less whole grains than normal weight individuals [2-6]; our study suggests that whole grains may improve the metabolic function of the

aberrant microbiota in obese individuals and improve health by ramping up short chain fatty acid production.

Although our study has shown that how different dietary fibers and whole grains can influence the gut microbiota community and metabolism, our knowledge in this area is still limited. More research at the genome level should be conducted to establish the relationships among dietary fiber and whole grain utilization, the growth of certain bacterial taxa, and metabolic diseases including obesity.

Reference

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APPENDICES

Appendix A. Selected SAS Code

A.1.SCFA for Method 1 and Method

SAS software (version 9.2, SAS Institute, Cary, NC) was used to determine statistical differences, which were defined as $P<0.05$, between gas, pH, short chain fatty acids (SCFA), and branched chain fatty acids (BCFA) during fermentation. Data were analyzed using a mixed model analysis of variance (PROC MIXED) with df (dietary fiber) ,group as main effects. SCFA and BCFA were analyzed for each time point(6h,12h, and 24h) ANOVA was used.

```
data example A1(butyrate);
input df group ace but pro scfa ibu iva bcfa gas;
datalines;
[data]
;
proc glm;
class df group;
model but = df group df*group;
means df group df*group;
lsmeans df group df*group / pdiff lines;
run;
```

A.2.SAS was used to determine statistical differences, which were defined as $P<0.05$, of the shift of bacterial taxa (282) between different treatments (dietary fibers and control). ANOVA was used followed by with Tukey's multiple comparison adjustment

```
data classifier_TC;
input sample $ trt $ X1-X282;
cards;
[data]
;
```

```
proc print data = classifier_TC;
run;
proc glm data = classifier_TC;
class trt;
model X1-X282 = trt;
lsmeans trt / pdiff lines adjust = tukey;
run;
```

A.3.Difference of Unifrac Index

Repeated ANOVA (Graphpad grism 5.1) was used to determine the difference between different dietary fibers.

A.4.difference of gut microbiota between fecal samples

T-test was used to determine the statistical differences of the microbial compositions between obese and normal weight individuals.

A.5. Difference of gut microbiota between obese and normal weight group after dietary fibers treatments

T-test was used to determine the statistical differences of the microbial compositions between obese and normal weight individuals.

A.6. Correlation between SCFA and bacterial taxa, CHO utilization and bacterial taxa

```
Data example A6;
Input A6 X1-X282;
Datalines;
[data]
;
Proc corr;
var butyrate X1-X282;
Run;
```

Appendix B. Detailed Methodology

B.1. Total dietary fiber / carbohydrate assay [1]

1. Weigh up to 300 mg of sample to contain about 10-20 mg total dietary fiber and not more than 180 mg starch into a 15 ml screw cap test tube.
2. Add 3 ml of acetate buffer (0.1 M, pH 5, containing 5 mM $CaCl_2$) and 25 µl of thermostable α-amylase (Sigma A-9505 or Megazyme); cap; mix; heat to boiling for 1 h.
3. Cool; add 0.140 ml of amyloglucosidase (Sigma A-7095, 300 U/ml); incubate at 60 °C overnight.
4. Cool; add 12 ml absolute ethanol; mix; leave for 1 h in an ice bath.
5. Centrifuge at 800*g* for 10 min; discard supernatant; wash pellet by suspending and re-centrifuging with 80% ethanol (2 × 20 ml) and then with acetone (2 × 15 ml).
6. Insert glass rod in test tube and mix occasionally while pellet dries; allow pellet to dry completely overnight in the hood;
7. Add 0.3 ml of 12 M sulfuric acid; let stand at 30 °C for 1 h; turn water bath to 40 °C.
8. Add 1 ml of myo-inositol (amount of inositol added should approximate the average of the individual sugar contents, about 3 mg/ml) and 7.4 ml of water; add water in such a way as to wash the glass rod of any remaining residue and remove glass rod; make sure there is no residue clinging to the side of the test tube.
9. Pressure cook tubes on HIGH for 1 h.
10. Filter hot hydrolysate through a dry, tared, frittered glass crucible into a 25 ml volumetric flask thoroughly rinse the tube and crucible with water; make sure to transfer all of the solids to the glass crucible; this is the hydrolysate syrup (H).
11. Dry crucible in oven at 105 °C for 16 h (or overnight); re-weigh ($_{Wi}$); ash in furnace at 500 °C for 1 h (place crucible in cold furnace, heat to 500 °C at 10 °C/min, hold at 500 °C for 1 h,

cool to 100 °C at 3 °C/min); remove from oven and cool completely in a dessicator before weighing ($_{wf}$).

a. $KL = \frac{(W_i - W_f) \times 100\%}{S}$

b. All weights are in mg

12. Add 0.357 ml of hydrolysate syrup (H) to a 35 ml tube; add 71 µl of 12 M ammonium hydroxide (8 ml of 30% ammonium hydroxide diluted to 10 ml); mix; add 36 µl of freshly prepared 3 M ammonium hydroxide (2 ml of 30% ammonium hydroxide diluted to 10 ml) containing 150 mg/ml sodium borohydride; add 5 µl 2-Octanol; incubate at 40 °C for 1.5 h.
13. Add 36 µl of glacial acetic acid; mix; add 0.5 ml of 1-methylimidazole; mix; add 5 ml of acetic anhydride; mix; let stand 10 min at room temperature.
14. Add 1 ml absolute ethanol; let stand 10 min at room temperature.
15. Move tubes to a cooler with ice up to shoulder of tube.
16. *Slowly* add 5 ml of well mixed 7.5 M sodium hydroxide; mix; add another 5 ml of 7.5 M sodium hydroxide.
17. Transfer ethyl acetate (top) layer to a fresh tube; make sure not to transfer any aqueous phase to tube; if so, dry with anhydrous sodium sulfate and transfer to another tube.
18. Separate alditol acetates using the following conditions:
    a. Injection volume: 1 µl
    b. Inlet temperature: 240 °C
    c. Carrier gas: He @ 2 ml/min
    d. Split ratio: 1:20
    e. Column: Elite 225,PerkinElmer N9316177, 30m×0.25mm×0.25µm
    f. Temperature program: Isocratic @ 220 °C for 25 min
    g. Detector temperature: 240 °C

19. Quantify alditol acetates relative to inositol peak using correction factors obtained from known standards (arabinose, xylose, mannose, glucose, galactose)

a. $CF_{MS} = \frac{A_{Std} \times W_{MS}}{A_{MS} \times W_{Std}}$

b. $MR = \frac{CF_{MS} \times A_{MS} \times W_{Std} \times F_m \times 100\%}{A_{Std} \times S}$

c. F_m= 0.88 for pentoses; 0.9 for hexoses; S = sample weight in mg

20. Add 125 µl of hydrolysate syrup (H) or 125 µl of galacturonic acid monohydrate (150 mg/ml; Std) to a 5 ml screw cap tube; add 125 µl of NaCl/boric acid (2 g NaCl and 3 g boric acid/100 ml); add 2 ml of concentrated sulfuric acid; mix *immediately*; cap; incubate at 70 °C for 40 min.

21. Cool; add 100 µl of freshly prepared 3,5-dimethylphenol (100 mg/100 ml in glacial acetic acid); mix several times over 5 min.

22. Read absorbance at 400 and 450 nm.

23. Calculate uronic acid content

a. $UA = \frac{A_H \times 150}{A_{Std}}$

b. $UR = \frac{UA \times F_u \times F_c \times 100\%}{S}$

c. F_u = 0.83 (monohydrate to residue); F_c = 0.81 (adjustment for absorbance of free uronic acid vs polyuronic acid)

24. TDF = UA + UR + KL

Notes: Total carbohydrate content (except Inulin): start from step 1(weigh 50mg samples), then add 0.3 ml of 12 M sulfuric acid, continue with step 8.

B.2. *In vitro* digestion

The *in vitro* digestion was followed by [3] with some modifications.

1. Whole grain flours and resistant starch-2 (25 g) were boiled for 20 min with 300 ml distilled water in a 500 ml beaker inside of another larger beaker that was filled with boiling water.

2. After cooling to room temperature, ~8 ml of 1M HCl were added to the sample to reduce the pH to 2.5. Ten ml of 10% (w/v) pepsin (P-700, Sigma, St. Louis, MO USA) dissolved in 0.05M HCl was added and the mixture placed on an orbital shaker (150 rpm) at 37 ℃ for 30 min to achieve the gastric phase.

3. The small intestinal phase was initiated with the addition of 50 ml 0.1 M sodium maleate buffer (pH=6, containing 1 mM $CaCl_2$) and ~20 ml of 1 M $NaHCO_3$ to bring the pH to 6.9. Fifty ml of 12.5% (w/v) pancreatin (P-7545, Sigma) dissolved in sodium maleate buffer and 2 ml of amyloglucosidase (3260 U/ml; Magazyme) were then added and samples were incubated in shaking water bath at 37 °C for 6 h.

4. Digested contents were then poured into dialysis tubing (molecular weight cut off 12,000-14,000) and dialyzed for 3 d against distilled water with changing of the water every 12 h. The retentate was then frozen (-20 ℃) overnight and then freeze dried.

B.3. *In vitro* fermentation

1. The method was according to (3). Firstly, prepare phosphate buffered saline solution, separate them in 5 bottles and autoclave them with then store them in the anaerobic cabinet.

2. Make nutrient medium in a 1 L beaker, autoclave it and add cysteine hydrochloride, vitamin K, and hemin in the anaerobic cabinet. Then divide into 5 sterile containers.

Checklist for autoclave:

a. Five bottles with buffer (cap slightly).

b. Five bottles with nothing.

c. 1L bottle with medium inside.

d. Three 500ml flasks with AL foil cover on them.

e. Cheesecloth.

3. Weigh 40mg/100mg carbohydrate into each tube. Include 2 blanks for each fecal preparation (blanks do not contain any sample in tube).

4. Move the tubes to the anaerobic cabinet, add 4 ml/10ml nutrient basal medium (w/v=1%) to the tube. Cap the tubes and store them at 4 ℃ for 12 h.

5. Mix 5g fecal sample with 50ml buffer (w/v=10%) in the anaerobic cabinet using the 0.00 balance. Mix for 1 min on the blender. Filter the solution through 4 layers of cheesecloth into a clean flask.

6. Inoculate each tube with 0.4ml/1ml of the solution above into each tube. Vortex the tubes and cap them, then put them in the shaking water bath (37 ℃) in cereal lab for 12h.

7. At the end of 6, 12 h, and 24h, plunge tubes into an ice bath to slow bacterial metabolism. Allow to cool for 5 min. Transfer exactly 1 ml of fermentation slurry into micro centrifuge tubes (5 tubes). Swirl tubes while taking samples to ensure an even draw of liquid and solid material into each microfuge tube. Store them in well-marked freezer boxes in the -80 ℃ freezer.

Notes:

1. The buffer solution contains resazurin. This is an indicator of anaerobic conditions. The indicator is colored (blue or pink) when oxygen is present and colorless when oxygen is not present. Before mixing your medium with samples, there should be no color from the resazurin (color of reduced medium will probably be pale yellow).

2. When preparing the buffer, the cysteine hydrochloride, vitamin K, and hemin should be added AFTER autoclaving. Therefore, you will have to add these in the anaerobic hood. For this reason, it might be best to keep the medium in 1 container until you add the last components and then divide into 5 sterile containers (autoclave 5 empty Erlenmeyer flasks with Al foil over opening).

3. Do not autoclave blender container. This will probably melt it. Just wash the containers well, soak in 10% bleach, and rinse.

4. Cut the tip off of blue pipette tips to take samples. This will prevent particulates from clogging up the pipette tip

5. Only adjust the pH of the phosphate buffered saline solution, NOT the medium. It might be a good idea to check the pH of the medium, though. It should be close to 6.8 (actually it was closed to 7.4 in our experiment).

Phosphate buffer	g/L	Nutrient medium	
NaCl	8	peptone water	2 g/L
KCl	0.2	yeast extract	2 g/L
Na_2HPO_4	1.15	NaCl	0.1 g/L
KH_2PO_4	0.2	K2HPO4	0.04 g/L
		$MgSO_4 \cdot 7H_2O$	0.01 g/L
		$CaCl_2 \cdot 2H_2O$	0.01 g/L
		$NaHCO_3$	2 g/L
		hemin	0.005 g/L
		L-cysteine hydrochloride	0.5 g/L
		bile salts	0.5 g/L
		Tween 80	2 ml/L
		vitamin K	10 μL/L
		resazurin solution	4 mL of 0.025% (w/v)/L

B.4.SCFA assay

1. Dissolve 20mg 4-methy valeric with 8ml water and add 2ml metaphosphoric acid (25% w/v) into it.
2. Dissolve 20mg 4-methy valeric acid, 20mg acetate acid, 20mg butyric acid, 20mg isobutyric acid, 20mg propionic acid, 20mg valeric acid and 20 mg iso-valeric acid with 8ml water , add 2ml metaphosphoric acid(25% w/v) into it .
3. Add 0.2 ml solution in step 1 to 1 ml sample.

4. Add 0.2ml solution in step 2 to 1 ml water.
5. Centrifuge at 16000rmp for 10min.
6. Transfer the supernatant to a clean tube.
7. Measure with GC.

B.5.References

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Appendix C. Project Proposal.

Ability of Specific Dietary Fibers to Normalize

The Gut Microbiota in Obese states

Summary:

Current studies show that obese individuals harbor an abnormal gut microbiota (dysbiosis) that supports an increased capacity for harvesting energy from food. Considering that different dietary fibers and whole grain cereals have distinct chemical compositions and influence on the gut microbiota, a specific whole grain cereal or dietary fiber may most effectively alter the microbiota from the obese state and thus serve as an innovative strategy to treat obesity. We will subject several different kinds of whole grain cereals and dietary fibers to *in vitro* digestion and fermentation so as to imitate what happen in human gastric intestinal tract. Short chain fatty acid (SCFA) and gas production will be quantified and the shift of bacteria will be assessed by pryosequencing. We want to know which of the dietary fibers is most effective at shifting the gut microbiota to a healthier state. The outcomes can serve as preliminary results to the clinical trial testing the effect of certain dietary fibers on obesity.

Justification and Objectives:

About one-third of U.S. adults (33.8%) are obese (Flegal et al 2010). Obesity is the key contributor to many metabolic syndromes worldwide such as hypertension, dyslipidemia, type 2 diabetes, colon cancer, cardiovascular disease and stroke (Alison et al 2001).

Human intestines harbor an immensely complex and diverse microbiota that can be viewed as a super metabolic organ (15 fold-more genome than our host genome) with about 10^{14} bacteria and Achaea, composed of approximately 1,100 prevalent species (Junjie Qin et al 2010). The gut microbota has been found to affect energy harvest from the diet and fat storage. Germ-free (GF) mice are protected from diet induced obesity, and upon gut microbiota colonization a significant increase (42%) of body fat content occurs (Fredrik et al 2004).

Gut microbiota composition has been associated with obesity. Decreased diversity of microbiota will certainly lead to a dysfunction of the whole gut ecosystem which will affect the stability of certain bacterial groups. Studies (Ley et al 2006, Zhang et al 2009 and Turnbaugh et al 2009) show that there is a shift between two bacterial phyla (Bacteroidetes and Firmicutes in obese state): a reduction of Bacteroidetes and a proportional increase of Firmicutes in the process of weight gain. Also compared with lean people, there are relatively less Bacteroidetes in obese people, which are increased with weight loss.

The gut microbiota can be influenced by diet, particularly by dietary fibers in the diet. Indeed, certain dietary fibers can favor the growth of one or a group of bacteria at the expense of others (Gibson et al 1995). Moreover, obese people consistently consume less dietary fiber than normal-weight individuals (Davis et al 2006). Therefore, we hypothesize that whole grain and dietary fibers may be an excellent way to treat the obesity by altering and modulating of the gut microbiota.

However, since whole grains and dietary fibers are actually composed of many chemical compounds with different physiological effects, we further hypothesize that different whole grains and dietary fibers will exhibit varying efficacy with respect to correcting the abnormal gut

microbiota in obesity. To test the hypothesis, we will use *in vitro* fecal fermentation to determine what kinds of whole grain and dietary fibers that can be most effective and efficient in altering the gut microbiota composition to a healthier state which may result in weight loss (Firmicutes:Bacteroidetes ratio and shift the obese microbiota toward that of normal weight individuals). The objectives of this study can deep our understanding of the relationship between diet, gut microbiota and obesity, which sever as the new way of weight loss.

Literature review:

Over 60% Americans are overweight and the prevalence of obesity increased dramatically in 1990s and a similar increase was seen in US children (Mokdad et al 1999). Despite the increased awareness of weight control, the population of obese people continues to increase according to the National Center of Disease Control & Prevention (Khan and Mokdad et al 2001). The change of diet may be novel approaches to treating obesity with high patient compliance due to minimal changes in physical habits.

Dietary Fiber and Obesity:

AACC International gives the definition of dietary fibers as “the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine.” It includes polysaccharides, oligosaccharides, lignin and associated plant substances (AACC). A common classification divides dietary fibers into two categories: insoluble dietary fibers consisting of cellulose, hemicellulose and lignin, and soluble dietary fibers containing pectin, gums, inulin, and some storage polysaccharides. Dietary fiber exhibits one or more beneficial effects either on laxation, blood cholesterol attenuation and blood glucose attenuation. Dietary fibers have been shown related to weight loss in many studies and the following part will discuss these studies and the mechanism of dietary fiber impacting on weight loss in detail.

Much research has been devoted toward assessing the relationships between dietary fiber consumption and obesity including both epidemical studies using food frequency questionnaires

(FFQ) and intervention studies based on randomized controlled trials (RCT). Cross-sectional studies show a significant inverse association between dietary fiber consumption and Body Mass Index (BMI) (Vijai et al and Trucker). In 203 healthy men, it was reported that intakes of complex carbohydrate and dietary fiber were associated with reduced body fat, especially in highest body fat groups (Lisa et al 1996). In another study, increasing dietary fiber consumption significantly reduced the risk of weight gain and fat in women (Trucker et al 2009).

In spite of the convincing role of dietary fiber in preventing obesity suggested by epidemiological studies, clinical trials on weight loss and dietary fibers intake are also inconsistent. For instance, a double-blind controlled trial designed with 32 women showed a significantly higher decrease in body weight in subjects taking guar gum compared with the placebo group (Tuomilehto et al 1980), while a meta-analysis of randomized placebo controlled trials suggests that guar gum is not efficacious for reducing body weight (Pittler et al 2001). More RCT with a larger subject population and long term treatments may help to draw a more firm association between dietary fiber intake and weight loss.

There is still much debate about the exact mechanism of the function for dietary fiber on obesity. There are three proposed mechanisms for prevention of obesity with dietary fiber: physiological properties, hormone modulation of dietary fiber and impact on gut microbiota.

1. Physiological Properties of Dietary fiber on obesity

The mechanism for prevention of obesity with dietary fiber can be stated as below: (1) On average, only 40% of dietary fiber are fermented in the colon and the capability of water binding properties for dietary fiber will lead to lower energy density compared with other refined carbohydrate matrix (Geraci et al 1993); The weight of food consumption for human is consistent (Roll et al) so high fiber food can displace energy and calories compared with high energy density food. (2) Increased dietary fiber consumption may have links to lower digestibility of fat and protein especially with high fat diet. Dietary fiber decreases the metabolizable energy content and nutrient digestibility of mixed diets fed to humans (Baer et al 1997). However, the SCFA

produced by the digestion of dietary fiber salvages some energy that may offset the effect stated above. Despite the offset, consumption of dietary fiber still brings about a small additional loss of energy intake. (3) Food containing dietary fiber tends to have a longer chewing time which will lead to more saliva excretion. As a consequence, it will take a longer time to eat the food and increase the feeling of satiety. (Aleixandre et al 2008);

2. Hormone Modulation of Dietary Fiber.

To increase satiety and reduce huger, it is important to note that the satiety effect of dietary fiber may be administered by several gut hormones including insulin, leptin, ghrelin, cholecystokinin (CCK) and glucagon-like peptide 1 (GLP-1), which can regulate the energy balance. Ghrelin is an endogenous peripheral hormone that can induce hunger and increase food intake (1)Ghrelin plays an important role in decreasing gastric acid secretion and stimulating gastric emptying (Masuda et al 2000). The consumption of ghrelin in rats increases food intake and decreases lipid oxidation (Kojima et al 2002). Dietary fiber can impact on ghrelin level in two ways. On one hand, it has been shown that fiber consumption can reduce the plasma ghrelin level in a randomized, single-blind, controlled, crossover intervention trial (Djurhuus et al 2002). Another pathway that has been reported is that dietary fiber intake can increase GLP-1 level that can suppress ghrelin secretion.

Fermentable dietary fiber is shown to increased peptide-YY (PYY) levels in rodents (Greenway et al 2007).PYY is a gut hormone excreted by C-Cells and exerts impact mostly on lower gut intestine. PYY can reduce appetite in both obese and lean individuals (Batterham et al 2003). Fermentable dietary fiber is shown to increased PYY levels in rodents (Greenway et al 2007). However, other studies demonstrate that no significant differences have been found between lean and obesity individuals, suggesting that reduction of PYY level may be unrelated to obesity (Batterham et al 2003). Thus, it is still speculative whether there is relation between obesity and PYY.

GLP-1 is secreted by L-Cells distributed along the intestinal tract (Murphy et al 2006). It has been shown that peripheral administration of GLP-1 inhibits food intake and appetite both in human and rats through their peripheral effect (vagal nerve) and by acting directly on the arcuate nucleus (Druce et al 2004). It has been found that consumption of dietary fiber leads to prolonged increase of plasma GLP-1(Gee et al 1996). In addition, DPPIV is an enzyme dipeptidylpeptidase IV (DPPIV) that has been involved in suppressing the activities and cleaving the GLP-1 (Burcelin et al 2001). Fiber intake may also reduce the DPPIV activities by about 30%. Some studies show GLP-1 is low in obese people and weight loss will lead to normalize this level (Verdich et al). Other studies have not seen this shift (Visbll et al 2001). It is still not conclusive whether the GLP will affect obesity. More research is needed to access the relationship between dietary fiber and hormone modulation.

3. Gut Microbiota, Dietary Fiber and Obesity

The bacteria in our gut belong to 4 phyla including gram positive, Actinobacteria, Firmicutes, and gram-negative Bacteroidetes and Proteobacteria. The microbiota in our gut has been coevolving with us and shows many manifested physiological and pathological impact on the host. It allows us to extract extra energy dietary fiber because the gut microbiota can express enzymes (lycoside hydrolases and polysaccharide lysas) that we do not express (Sonnenburg et al 2005). Beyond that, the microbioa can also interact with the host in a symbiotic way that shapes and models our immune system and controls the regeneration of the intestinal epithelium (Lee et al 2010).

The gut microbota has been shown to impact energy harvest from diet and fat storage (Bäckhed et al 2004). Moreover, by transplanting caecal sample from lean mice and obese mice to GF mice for two weeks, it is observed that mice with the microbiota from obese ones gain more fat and extract more calorie when compared with the others (Peter et a 2006). Different extraction of calorie from ingested food may be attributed to different composition of bacteria. For instance, the microbiota in obese people prone to extract more energy. The metabolism of

bacteria will enhance energy extraction (Dibaise et al 2008). Thus, microbiota composition has a profound impact on weight regulation.

The underlying mechanism for the role of the gut microbiota in obesity may be explained using GF mice: (1) Fasting-induced adipocyte factor (Fiaf) which acts as an inhibitor for lipoprotein lipase (LPL) can be selectively suppressed by the gut bacteria. LPL can enhance the storage of fat storage process in liver (Bäckhed et al 2009). Fiaf is known as the angiopoietion-like protein selected in liver and intestines. This protein can act as an inhibitor to LPL which is a key regulator for fatty acid release and adipocyte triglyceride accumulation. Interestingly, by comparing GF wild-type and Fiaf/knockout mice when fed with a western diet using same protocol, Fiaf- deficient animals appear to gain more weight than the corresponding wild-type mice. (2) Changing the level of AMP-activated protein kinase (AMPK).The AMPK plays a key role in regulating cellular energy metabolism. Increasing the AMPK lever will promote the fat oxidation process. Research from mouse study found a significantly high level of AMPK in the liver of GF mouse compared to the conventional mouse. It is also noted that maybe a higher level of NADH in the livers of germ free mice will activate the AMPK (Rafaeloff-Phail et al 2004).In addition; AMPK can be regulated by ADP. A fall in intracellular ATP levels can activate the energy producing pathway (Xiao et al 2011).

Studies show that there is a shift between the two divisions (Bacteroidetes and Firmicutes): a reduction of Bacteroidetes and a proportional increase of Firmicutes with the process of weight gain (Ley et al 2006 and Turnbaugh et al 2006).It is also strikingly noted that compared with lean people, there is relatively less Bacteroidetes in obese people and accompanied with the weight loss, there will a substantial increase of Bacteroidetes with low calorie food. Although some other studies have not found significant difference between Firmicutes and Bacteroidetes (or the opposite), it is notable they have also found that obese individuals have distinctly different intestinal communities than normal-weight individuals (Zhang et al 2008).

The short term change in some bacterial groups may be of great importance. From an ecological perspective, the alteration of certain bacteria by themselves in the long term will cause the change of relative population of many other groups through competing with the ecological niche which will impact on the metabolism status.

Using both dietary and physical intervention, there are significant microbiota changes between high weight loss group and the low weight loss group. For the total bacteria, *B. fragilis* group and *Clostridium leptum* group, and *Bifidobacterium catenulatum* group counts were significantly higher ($P < 0.001–0.036$) while levels of *C. coccoides* group, *Lactobacillus* group, *Bifidobacterium, Bifidobacterium breve*, and *Bifidobacterium bifidum* were significantly lower ($P < 0.001–0.008$) in the high weight–loss group than in the low weight–loss group due to the intervention (Santa Cruz et al 2009).

To summarize, although many studies have been conducted to prove the relationship between whole grain food and dietary fiber intake with obesity, the results are controversial and the underlying mechanism remains unclear. Recently, metabolic disorder has been linked to the gastrointestinal microbiota. The connection between gut microbiota and weight gain may give an innovative strategy for the prevention and treatment of obesity. Dietary intervention (whole grain and fibers) has been shown to reshape and modulate the gut microbiota and the specific kind of dietary intervention is of great value and significance which can modulate gut microbiota most effectively to promote a lean state.

Experimental Design and Procedures:

Seven obese and six normal weight people based on the BMI will be identified from the patient database at Rush University Medical Center. Stool samples were collected from both obese and normal weight people without any known gastrointestinal disease or antibiotic consumption in the past 6 months. Fecal samples were collected through a specimen hat inserted under the toilet seat and mailed to UNL in Anaerocult C bags with oxygen absorbers on dry ice. The fecal samples were stored under -80 degree before analysis.

1. In vitro digestion

In vitro digestion was performed using a variety of cereal grains (corn, wheat, rye, rice and oats) and purified dietary fiber (resistant starch type-2, beta-glucan, inulin, corn arabinoxylan (AX), pectin and guar gum). Beta-glucan and AX were purified in the lab. Beta-glucan was extracted as in Irakli et al. (*J. Sci. Food Agric*. 2004, 84, 1170-1178), except beta-glucan was precipitate with ethanol rather than ammonium sulfate. AX was extracted by treating corn bran with Termamyl, Neutrase, and AMG followed by 1 M NaOH. The extracted AX was bleached with hydrogen peroxide and precipitated with ethanol and dried with acetone. The *in vitro* digestion was followed by Mishra et al (*J of Cereal Science* 50(2009) 61-66) with some modifications: the cereal grain samples (25g) were treated with 50 ml 12.5% α-amylase and 2ml AMG and the digestion process was increased to 6 h. The reason why we made this modification is that in the preliminary study with the enzyme concentration stated in the paper, starch cannot be effectively removed and the remaining starch in the sample is high which may affect the dietary fiber fermentation. This process mimics food digestion in human gastrointestinal tract which contains a gastric phase and small intestinal phase. *In vitro* digestion was followed by a dialysis process in which the monosaccharide produced during the digestion and protein hydrolysates were removed. Freeze drying of the dialysate was the last step to obtain the samples ready for *in vitro* fermentation. The dried samples were stored under -20 degree before analysis.

Total starch assay of whole grain sample after *in vitro* digestion (AACC DMSO method) was performed to check whether starch was digested during the *in vitro* digestion process. Total carbohydrate assay is used for both whole grain sample and purified dietary fiber sample. Total carbohydrate content was assessed based on the neural sugar and uronic acid according to the AACC 32-25 method .For inulin sample, since the fructan will degrade during acid hydrolyze step. The carbohydrate content will be measured with AACC method 32-32 and AOAC Method 999.03.

2. In vitro fermentation

Small scale 12 h *in vitro* fermentation was completed using different weight (but with the same total carbohydrate content) of cereal grain and dietary fiber samples after *in vitro* digestion. This process is followed in Sanz et al (*J.Agri.Food Chem*.2005, 53, 5192-5199) with a larger sample size. Gas production and short chain fatty acid data is measured and analyzed with SAS software. SAS was used to see the relationship between BMI, SCFA production and gas production. SCFA were quantified based on the protocol described by Campbell and coworkers (*J Nutr 1997,* 127:130–136) with small modification .Briefly, 1ml culture sample were removed from storage at -80°C and thawed on ice. The samples were added with 0.25ml 5 %(w/v) metaphosphoric acid containing 5-10 mM 4-methylvaleric acid and vortexed, followed by centrifugation for 10 min at 16,000 rpm. The supernatant was stored overnight at -20°C. Next, samples were thawed and centrifuged in the same conditions as before. SCFA were quantified by gas chromatography.

3. Microbial composition

Microbial composition was assessed before *in vitro* fermentation and after *in vitro* fermentation by deep pyrosequencing of 16S ribosomal RNA genes amplified by PCR from the community in the Core for Applied Genomics and Ecology (CAGE).

4. Total carbohydrate measurement

Total carbohydrate content remaining in the culture slurry after *in vitro* fomentation will be quantified to determine the consumption of each dietary fiber during the fermentation process. Since different dietary fibers have quite distinctive chemical compositions and physiological effects, specific bacteria may have unique mechanism of consuming certain types of dietary fibers more efficiently. The data will also be used to form hypotheses describing the relationship

between dietary fiber structure and the gut microbiota and obesity, which will be the basis for future research.

Expected Outcomes:

The most important and primary outcome will be the shift between Firmicute and Bacteroidetes after 12 h of *in vitro* fermentation compared with the bacterial composition before in vitro fermentation using certain dietary fibers in obese groups. The difference of bacterial composition between obese and normal weight individual will be as another outcome. SCFA data will be used to assess the different fermentation profile of different groups. Thus, specific dietary fibers can be found which are mostly effective in shifting the obese type bacterium to a normal type one.

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